Dissertation
submitted to the
Combined Faculty of Natural Sciences and Mathematics
of the Ruperto Carola University Heidelberg, Germany,
for the degree of
Doctor of Natural Sciences

Presented by
Lukas Frese, M.Sc.

born in: Göttingen

oral examination: March 30, 2023

Population relevance of neurotoxic effects in refined and alternative behavior tests with zebrafish (*Danio rerio*)

Referees: Prof. Dr. Thomas Braunbeck
Prof. Dr. Gert Fricker

Acknowledgements

I would like to try and remember to thank everyone who has supported me during the creation of this work and especially during the writing phase (which took more than a little longer than anticipated…)

First of all, thanks to Prof. Dr. Thomas Braunbeck for re-motivating me at difficult points during the writing process with his sober analyses and affirmation of the progress that had already been made but sometimes escaped from view. Under his supervision, I had the privilege of working on my own project with a lot of conceptual freedom while always knowing I could rely on his feedback if something did not work out as intended. But already way before this thesis, he has provided a major part of my scientific field, laboratory and teaching experience and provided the zoology & broader ecological education that put me in the position I am in today, for which I am deeply grateful.

Thanks to the entire NeuroBox cluster and especially the late Dr. Tamara Grummt for developing such an important project and the exchange and valuable input during our meetings. Likewise, I would like to thank the remaining members of my Thesis Advisory Committee, Prof. Dr. Stephan Frings and Prof. Dr. Gert Fricker, for their open ears and suggestions on my path towards a functional method.

I have been very lucky to have worked with great people in AG Aquatox who could be counted on to not only support one other with ideas and to make sure there was always enough fish (eggs) to go around, but to lighten up even early weekend mornings with their company: Dr. Lisa Baumann, Dr. Katharina Brotzmann, Maria Fischer, Lisa Gölz, Dr. Lisa Hanslik, Dr. Patrick Heinrich, Dr. Nadine Kämmer, Dr. Ann-Kathrin Lörracher, Susanna Mieck, Dr. Pauline Pannetier, Carola Leitner, Dr. Rebecca von Hellfeld and Dr. Florian Zindler. And of course, special thanks to the container crew for the best workplace decorations and humor!

Finally, I owe to biggest gratitude to my family, especially Gesa for her support and ultimate patience and/or the repeatedly necessary shoves back to the writing table during the difficult past two years.

Previously published data

Contents of the research outlined in this thesis have been published. No publications are given in their entirety or in place of a particular chapter. However, the original version of the manuscript, which was conceptualized and written entirely by me has been incorporated into chapter 5 of the present work:

> **Frese and Braunbeck (2022):** Adapting classic paradigms to analyze alterations of shoal-wide behavior in early-life stages of zebrafish (*Danio rerio*) – A case study with fluoxetine. Neurotox Teratol 95, 107136. https://doi.org/10.1016/j.ntt.2022.107136

In accordance with the copyright policy of Elsevier, I am entitled to use this manuscript for non-commercial purposes. This includes the preparation of a thesis.

For further information, please refer to:
https://www.elsevier.com/about/policies/copyright/permissions

Chapter 7 contains data from the following bachelor theses, which were designed and supervised be me, while experiments were performed jointly with the students. Data evaluation, presentation and discussion were carried out exclusively by me:

> **Lisa Stötzel (2021):** Effects of suspected neurotoxin exposure on the swimming behavior of zebrafish (*Danio rerio*) larvae
>
> **Marius Schubert (2021):** Histological analysis of zebrafish larvae (*Danio rerio*) exposed to low concentrations of neurotoxins

List of non-standard abbreviations

SI units are not included.

AChE	acetylcholine esterase
ATSDR	Agency for Toxic Substances and Disease Registry (USA)
CAD	computer-aided design
CBZ	carbamazepine (5*H*-dibenzo[*b*,*f*]azepine-5-carboxamide)
DMSO	dimethyl sulfoxide
dpf	days post-fertilization
EC_{10}	10% effect concentration
ELS	early-life stage toxicity test (OECD TG 210)
FET	fish embryo toxicity test (OECD TG 236)
FLX	fluoxetine ((RS)-*N*-Methyl-3-phenyl-3-(4-trifluoromethylphenoxy) propylamine)
FSTRA	fish short term reproduction assay (OECD TG 229)
GOW	*Gesundheitlicher Orientierungswert* (→ HRIV)
HNTC	highest non-teratogenic concentration (nearly equivalent to → EC_{10})
hpf	hours post-fertilization
HRGV	health related guidance value (German: “LW”)
HRIV	health related indication value (German: “GOW”)
LOEC	lowest observable effect concentration
LTLL	long-term, low-level
LW	*Leitwert* (→ HRGV)
NOEC	no observable effect concentration
NOEL	no observable effect level
OECD	Organisation for Economic Co-operation and Development
PXM	paraoxon-methyl (dimethyl (4-nitrophenyl) phosphate)
TDCPP	tris(1,3-dichloroisopropyl) phosphate
TG	test guideline
UBA	*Umweltbundesamt* (German environmental protection agency)
UHP	upper half preference

Table of contents

1. Abstract

Water supplies are widely, but unobtrusively contaminated with numerous substances of largely unknown biological properties. A particularly worrisome group are neurotoxic substances, which may, in the long term, not only affect human health, but also wildlife. Neurotoxic effects have become an issue of emerging concern in ecotoxicology, since they may have multiple underlying mechanisms, are often hard to detect, but have the potential to give rise to a severe adverse outcome. As neurotoxicity is even more difficult to detect without extensive animal testing, it presents a major challenge to modern ecotoxicology which is striving to reduce and replace animal studies.

My model species, the zebrafish (*Danio rerio*), is widely used in aquatic ecotoxicology but room for refinement remains especially where tests are carried out with adult individuals instead of potentially less perceptive early-life stages. Since zebrafish, like many other small fish, naturally form shoals and likely behave differently in isolation, I developed a shoal-based approach. In brief, early-life stage tests according to OECD TG 210 were augmented by two behavior tests that are typically carried out with single adult fish, but could be adapted to groups of juveniles with acceptable limitations: a novel tank test and a predator response assay. The selective serotonin reuptake inhibitor fluoxetine ((RS)-*N*-Methyl-3-phenyl-3-(4-trifluoromethylphenoxy) propylamine) served as model substance during a proof-of-concept study. In a follow-up study, I verified the suitability of this approach using a selection of other substances with different modes-of-action: carbamazepine (sodium channel inhibition), paraoxon-methyl (acetylcholine esterase inhibition), and tris(1,3-dichlorisopropyl) phosphate (TDCPP; endocrine disruption). Finally, in order to assess whether existing alternative methods correlate to immediately population relevant endpoints, I carried out several other experiments across the life-stages of zebrafish with the same model substances.

Fluoxetine produced adverse effects down to concentrations three orders of magnitude below the EC_{10} from acute fish embryo toxicity tests (OECD TG 236). The known neurotoxicants carbamazepine and paraoxon-methyl caused significant effects on zebrafish behavior both upon release into a novel tank and after presentation of a predator dummy. TDCPP, which is thought to disrupt neural development at much earlier stages than those exposed in my experiments, only caused minor behavioral changes. Histopathology of the test fish confirmed the absence of acute organ damage at the concentrations used (always $\leq EC_{10}$ from fish embryo tests). The suitability of shoal-based behavioral changes in juvenile zebrafish as sensitive endpoints of neurotoxicity could thus be confirmed. The deviations in behavior compared to the control groups permit conclusions about the "anxiety state", which arguably influences the fish's survival chances in the wild. An early and more abstract behavior endpoint, larval motility (6 dpf), also proved to be very efficient and held up well in a comparison with adult and juvenile behavior tests. Finally, a reproduction assay with adult fish exposed to fluoxetine revealed decreased fecundity as another directly population relevant effect of this chemical.

Correlation with embryonic and further adult data from literature revealed the good predictive power of 24-h spontaneous coiling tests for later behavior defects, leading me to propose a set of embryonic tests (FET + coiling) for neurotoxicity range-finding and screening in the future. If the results from these “alternative methods” are negative or inconclusive, *in vivo* testing is indispensable to assess neurotoxicity; as such, larval motility and juvenile behavior assays might follow.

2. Zusammenfassung

Unsere Wasserversorgung wird umfassend, aber unauffällig mit immer neuen Substanzen unbekannter Umweltauswirkungen kontaminiert. Eine besonders besorgniserregende Gruppe sind neurotoxische Substanzen, da sie nicht nur die Tierwelt, sondern langfristig auch die menschliche Gesundheit bedrohen. Neurotoxische Wirkungen sind in der Ökotoxikologie zu einem immer wichtigeren Thema geworden, da ihnen mehrere Mechanismen zugrunde liegen können und sie somit oft schwer nachweisbar sind, aber das Potenzial haben, schwerwiegende negative Folgen zu verursachen. Da Neurotoxizität ohne umfangreiche Tierversuche noch schwieriger zu erkennen ist, stellt sie eine große Herausforderung für die moderne Ökotoxikologie dar, die bestrebt ist, Tierversuche zu reduzieren und zu ersetzen.

Meine Modellspezies, der Zebrabärbling (*Danio rerio*), wird in der aquatischen Ökotoxikologie häufig verwendet, aber es gibt insbesondere bei Tests mit erwachsenen Individuen anstelle von potenziell weniger empfindlichen frühen Lebensstadien noch Raum für Verbesserungen. Da Zebrabärblinge, wie auch viele andere kleine Fische, von Natur aus Schwärme bilden und sich in der Isolation wahrscheinlich anders verhalten, habe ich einen schwarmbezogenen Ansatz entwickelt. Im Wesentlichen wurde der „*Early-life stage*“-Test gemäß OECD TG 210 durch zwei Verhaltenstests ergänzt, die normalerweise mit einzelnen erwachsenen Fischen durchgeführt werden, aber mit vertretbaren Einschränkungen an Gruppen von Jungfischen angepasst werden können: die Konfrontation mit einer neuen Umgebung (*„novel tank“*) und die Reaktion auf einen Räuber (*„predator response“*). Der selektive Serotonin-Wiederaufnahmehemmer Fluoxetin ((RS)-*N*-Methyl-3-phenyl-3-(4-Trifluormethylphenoxy)-propylamin) diente als Modellsubstanz für den Machbarkeitsnachweis. In einer Folgestudie habe ich die Eignung dieses Ansatzes anhand einer Auswahl anderer Substanzen mit unterschiedlichen Wirkungsweisen überprüft: Carbamazepin (Blockade von Natriumkanälen), Paraoxon-Methyl (Hemmung der Acetylcholinesterase) und Tris(1,3-dichlorisopropyl)-phosphat (TDCPP; Störungen des Hormonsystems). Drittens habe ich mehrere andere Experimente über die Lebensstadien des Zebrabärblings mit denselben Modellsubstanzen durchgeführt, um zu beurteilen, ob die vorhandenen alternativen Methoden mit den unmittelbar populationsrelevanten Endpunkten korrelieren.

Fluoxetin verursachte schädliche Wirkungen ab Konzentrationen, die drei Größenordnungen unter dem EC_{10}-Wert aus akuten Fischembryotoxizitätstests gemäß OECD TG 236 lagen. Die bekannten Neurotoxine Carbamazepin und Paraoxon-Methyl verursachten signifikante Veränderungen des Verhaltens der Zebrabärblinge, sowohl nach dem Aussetzen in ein neues Becken als auch nach der Präsentation einer Raubfisch-Attrappe. TDCPP, von dem angenommen wird, die neuronale Entwicklung in wesentlich früheren Stadien als in meinem experimentellen Zeitfenster zu stören, verursachte dagegen nur geringe Verhaltensänderungen. Histologische Untersuchungen der Versuchsfische bestätigten die Abwesenheit von akuten Organschäden bei den verwendeten Konzentrationen (immer $\leq EC_{10}$ aus Fischembryotests). Die Eignung von schwarmübergreifenden Verhaltensänderungen bei juvenilen Zebrabärblingen als empfindliche Endpunkte für Neurotoxizität konnte somit bestätigt werden. Die Verhaltensabweichungen im Vergleich zu den Kontrollgruppen lassen Rückschlüsse auf die "Ängstlichkeit" zu, die die Überlebenschancen der Fische in der freien Wildbahn beeinflussen dürfte.

Ein früher und abstrakterer Verhaltensendpunkt, die Schwimmfähigkeit von Larven (6 dpf), erwies sich ebenfalls als sehr effizient und hielt einem Vergleich mit adulten und juvenilen Verhaltenstests stand. Schließlich demonnstrierte ein Reproduktionstest mit erwachsenen Fischen, die Fluoxetin ausgesetzt waren, eine reduzierte Fruchtbarkeit als weitere direkt populationsrelevante Wirkung dieser Chemikalie.

Die Korrelation mit embryonalen und weiteren adulten Daten aus der Literatur offenbarte die gute Vorhersagekraft von 24-Stunden-Schwanzschlagtests für spätere Verhaltensdefekte, was mich dazu veranlasst, zunächst zwei Embryotests (FET + Schwanzschläge) für künftige Neurotoxizitätsuntersuchungen und Screenings vorzuschlagen. Wenn die Ergebnisse dieser „alternativen Methoden“ negativ oder nicht eindeutig positiv sind, sind *in-vivo*-Tests für die Erkennung von Neurotoxizität noch immer unerlässlich zu sein und die Tests zur Schwimmfähigkeit von Larven und Verhalten von Jungfischen sollten folgen.

3. Background

3.1 Toxic compounds in the environment

Anthropogenic chemicals are released into the environment in large numbers and volumes on various routes, but we know worryingly little about the effects of these xenobiotics on organisms and physiology, including human health (Brown et al., 2005, Stein et al., 2002). Not only is modern society producing an ever-wider array of chemicals, but the detection capabilities are also improving so that we keep becoming more aware of chemicals in undesired places (Grummt et al., 2018b, Williams et al., 2009, Zhang et al., 2008). Aqueous systems in particular act as accumulators of pollutants due to wash-off from agricultural land or improperly sealed landfills as well as the constant influx of partially treated residential and industrial wastewater (Santos et al., 2010).

Thankfully, acute exposures of an ecosystem to exceptionally high toxin concentrations, especially untreated waste release events, is becoming rarer due to regulatory measures stemming from growing societal and political awareness of the associated dangers (Sharma and Sanghi, 2012). Instead, prolonged exposure to low but rather constant concentrations of pollutants is now the typical scenario for the organisms in our water cycle – including humans consuming drinking water with traces of chemicals despite all treatment efforts (Zhang et al., 2008). Lower concentrations, however, are not to be underestimated: long-term, low-level (LTLL) exposure with certain substances appears to cause severe effects over time without any acute poisoning events (Jamal et al., 2002). Fish in particular, having a relatively long life span, may accumulate these substances over time and often suffer adverse effects at 10-fold lower concentrations than are found to be relevant in acute scenarios (Ahlers et al., 2006). Presently, the most prominently challenging pollutants with easily overseen chronic effects at low concentrations are endocrine disruptors and neurotoxic substances – I shall herein focus on the latter.

3.2 Neurotoxicity on the rise

Neurotoxicity is a matter of growing concern in light of an increasing incidence of neurodegenerative diseases, although it is uncertain whether environmental agents or the aging population is mainly responsible for this trend, and the large portion of known or suspected neurotoxicants in industrial emissions (Brown et al., 2005, Stein et al., 2002). For instance, the exact causes of Parkinson's disease are unknown, but environmental toxins including pesticides have been identified at least as contributors and chronic exposure to the widely used pesticide rotenone can cause a syndrome very alike Parkinson's disease in rats (Betarbet et al., 2000). Up to 75 % of anthropogenic xenobiotics have been suspected to be neurotoxic (Ton et al., 2006) and signs for adverse effects at concentrations far below the acute toxicity test results are amassing. Conclusive studies are still missing in many cases, but the picture is alarmingly clear for some substance groups like organophosphate pesticides (Jamal et al., 2002).

Children, infants and even fetuses are at increased risk due to the higher toxin susceptibility of developing brains without a mature blood-brain barrier and the possibility of exposure in utero (Andersen et al., 2000, Christensen et al., 2013, Stein et al., 2002).

Early childhood exposure may cause long-lasting effects: for instance, Eddins et al. (2010) demonstrated adverse learning effects of chlorpyrifos exposure during early zebrafish development to persist into adulthood. Adverse effects are manifold, ranging from attention deficits to IQ decline and increased probability of mood instability and delinquency later in life (ATSDR, 2020, Stein et al., 2002).

Unfortunately, many such effects are subclinical at the individual level and overarching trends and correlations with possible causes only become observable in hindsight (Grandjean and Landrigan, 2006). The "silent pandemic" caused by many years of leaded fuel use across the globe may serve as an example. Nowadays the link between childhood lead exposure and life-long adverse outcomes is nearly certain, but humanity is still struggling to replace the toxic fuel once and for all and residual exposure from reactivated dust particles to maternal transfer routes will accompany us for many years to come (ATSDR, 2020). Even low levels of environmental exposure have been revealed to cause measurable cognitive deficits (ATSDR, 2020). It has been estimated that approx. 0.2 – 0.3 IQ points are lost to any individual per µg/dL blood lead concentration, which accumulates to a major societal and economic impact when millions of people are affected (Grosse et al., 2002).

To prevent such an ignorance-born catastrophe from ever happening again, great care needs to be taken with all unknown substances as soon as they are identified and sufficient safety factors should be applied to acceptable concentrations to ensure the safe development of fetuses and children (Claudio et al., 2000). The practical problem for regulatory agencies, however, is that newly emerging substances in the water cycle are often largely unknown and require a rapid initial evaluation before there has been time for proper assessment.

3.3 Regulatory efforts and BMBF-sponsored research programs

Despite all measures to prevent and mitigate pollution, some substances persist through the processing steps of drinking water and need to be evaluated as soon as they emerge. The regulatory agencies are therefore faced with the challenge of rapidly assigning adequate safety thresholds for potentially lifelong consumption to largely unknown chemicals if they occur in drinking water at concentrations ≥ 0.1 µg/L or their structure suggests genotoxic activity (Grummt et al., 2018b). To enable such a quick response, the German environmental protection agency (*Umweltbundesamt*; UBA) has adapted the strategy of hierarchic test batteries that attempt to characterize the substance's potential effects. Based on the type of toxicity, different health related indication values (HRIV, Dieter 2014) are assigned that are thought to be within tolerable consumption limits (see Fig. 1). If exposure concentrations cannot reliable be kept below the HRIV, detailed testing is required in order to determine an evidence-based limit for safe consumption (health related guide value, HRGV) that is typically found to be higher than the HRIV (UBA, 2003). Thereby, HRIV test batteries replace many otherwise necessary animal studies or at least provide a precautionary estimate for dealing with the substance for the remaining months or years until these time-consuming studies can be concluded and/or verified.

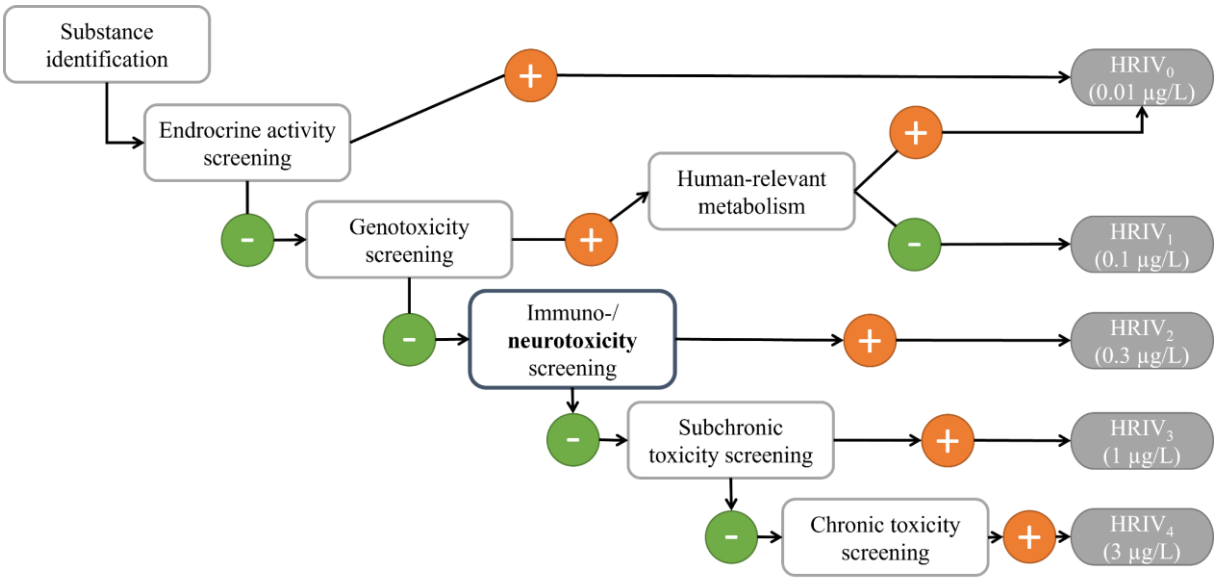

Fig. 1: Simplified schematic of the toxicity categorization process and resulting HRIVs (health related indication values) for a newly identified substance in the drinking water supply. "+" indicate specific effects have been found or data is missing, "-" indicates that the tests have found no effect. Only new substances that are detected at concentrations above 0.1 µg/L or possess structural properties indicating increased risk are processed in this manner. Each "screening" step represents a battery of several tests which have been described in detail by Grummt et al. (2018b) and are still under development, including contributions from this study toward in vivo neurotoxicity screening.

The generic precautionary value if no data is available at all is $HRIV_1$ (0.1 µg/L). The same value is ordinarily applied if genotoxicity is found during the screening process. Substances that are suspected to develop strong genotoxicity through activation in the human metabolism or affect the endocrine system are of particular concern and receive the strictest indication value ($HRIV_0$; 0.01 µg/L) as there are examples of such substances with adverse effects in extremely low doses. Higher concentrations are tolerated if these first screenings find no specific toxicity: substances that are untested or tested positive for neurotoxicity are assigned $HRIV_2$ (0.3 µg/L), those untested or positive for subchronic effects are assigned $HRIV_3$ (1 µg/L) and those untested or positive for chronic effects are assigned $HRIV_4$ (3 µg/L) until final guide values are determined by extended testing (Grummt et al., 2018b). Please note that a slightly different numbering scheme of the HRIVs has temporarily been used, e.g. by Grummt et al. (2013) and Dieter (2014), with index numbers according to the chronological order of HRIV definitions rather than the associated concentrations.

Let us take an exemplary look at $HRIV_2$ to understand how these thresholds came to be: If neurotoxicity is proven or not disproven by the screening process, up to 0.3 µg/L of the questionable substance is tolerated in drinking water and deemed safe for consumption because there are no known specific neurotoxins that are effective at such a low concentration. Rather, Kroes et al. (2000) found the no observed effect level (NOEL) of their most toxic test substance with neurotoxicity as its most sensitive endpoint (Ethyl-*p*-nitrophenyl phenylphosphorothioate) was 0.01 mg per kg bodyweight per day.

Assuming the WHO estimation of a daily water consumption of 2 L for a 60 kg adult (World Health Organization, 2011), this person could routinely consume water with up to 0.3 mg/L of the toxin while maintaining the NOEL and thus suffering no ill effects to the best of current knowledge. The indication value of 0.3 µg/L is conservatively set 1000-fold lower than this presumable maximum safe concentration. By the same calculations, a child with 10 kg bodyweight and an assumed consumption of 1 L of water per day may safely drink water up to a contamination with 0.1 mg/L of the chemical. The resulting safety factor of approx. 333 between $HRIV_2$ and this "worst case" NOEL can still be considered a reasonably safe margin of error.

Assuming we have indeed found the actual worst cases of drinking water relevant chemicals by now and the HRIVs are therefore sufficiently below dangerous levels (Dieter, 2014), the practical difficulty that remains is reliably determining each new substance's toxicity category. In order to act quickly after discovery, regulatory bodies require simple and fast screening methods. However, it is not trivial to make sure they are sensitive enough to detect all problematic substances without overestimating toxicity with ultimately irrelevant endpoints.

As briefly mentioned above, the recently concluded joint research project "ToxBox" targeted this problem by means of hierarchic test batteries that should serve to identify a chemical's toxicity category (Grummt et al., 2013). In such a battery, each test in a preliminary level may detect a different toxicity mechanism in each category, e.g. different neural cell lines shed light on adverse effects on proliferation, neurite growth or the formation of reactive oxygen species (ROS; Grummt et al., 2020). When one testing level gives rise to concerns, evaluations at a higher level of complexity (i.e., translatability) follow – up to the ultimately necessary validation through *in vivo* tests before $HRIV_2$ may be assigned (Grummt et al., 2013, Grummt et al., 2020). ToxBox was successful in laying out a multitude of promising new *in vitro* and *in vivo* tests with proofs of concept, but the neurotoxicity detection methods turned out to require additional attention.

Therefore, "NeuroBox" was designed as a follow-up to ToxBox focused on further developing and validating combined neurotoxicity tests in the light of the evasive nature of many neurotoxic mechanisms and increasing case numbers of neurodegenerative diseases (Kuckelkorn et al., 2020). As before, our work group contributed *in vivo* research with zebrafish (*Danio rerio*) to the joint project. Whereas single organs, especially neuromasts of the lateral line organ, and the enzyme acetyl choline esterase (AChE) were of primary interest during ToxBox (Grummt et al., 2018b, Stengel et al., 2017b), the scope was now extended to include the relevance of further findings and I was tasked with the subproject of evaluating the population relevance of effects seen in early life stages of zebrafish.

3.4 Zebrafish as a model

Our selection of zebrafish as a model species is not arbitrary, of course: the small cyprinid and especially its eggs is a popular model organism for ecotoxicity testing (e.g. Lammer et al., 2009, Scholz et al., 2008, Weigt et al., 2011). This is explained with several practical advantages: due to their size, zebrafish are easy to keep in relatively high densities; they are hardy and undemanding in terms of habitat (Di Paolo et al., 2015, Engeszer et al., 2007). Best of all, as a tropical species they are capable of breeding year-round for a steady supply of eggs, of which a healthy female can lay several hundred per week, enabling frequent high-throughput screens (Braunbeck et al., 2005, de Esch et al., 2012, Parichy, 2015). Furthermore, the eggs are transparent so the developing embryo can be observed from the point of fertilization without potentially falsifying the effective exposure concentration through dechorionation (Parichy, 2015).

These advantages of zebrafish embryos are particularly valuable from an ethical standpoint as they allow researchers to reduce the need for conventional animal tests. Studies using only embryos or eleutheroembryos (hatched, but not yet feeding) fulfill the replacement criterion according to Russell & Burch's famous "3R" principle as fish are not considered protected animals under EU law until the onset of independent feeding after approx. 120 h (Russell and Burch, 1959, Strähle et al., 2012). A prime example for a widespread replacement of adult fish with embryos for regulation purposes is the fish embryo acute toxicity test (FET; OECD, 2013b). Instead of testing for acute toxicity in adults, it can already be assessed with astonishing reliability within the first 96 h of development (Lammer, 2009).

When it comes to specific targets, alternative methods at the enzyme level are especially interesting from an economic view as they can be applied to many substances with potentially very high throughput. However, resilience effects may make the actual organismic much less sensitive, i.e. an effect on a very sensitive molecular target does not necessarily correlate to a downstream organismic response (Kroes et al., 2000). Cell culture experiments translate better and still allow for the screening of many cellular neurotoxicity mechanisms, but unfortunately the conclusive analysis of important effects that alter signal transmission or even more complex mechanisms within the nervous system such as behavior requires living organisms (Braunbeck et al., 2015, Stengel et al., 2017b).

Once again, the zebrafish embryo might come to the rescue: it already shows certain, albeit immature, behaviors that could serve as endpoints in a new test battery. The main question that remains is how much can be interpreted into a mere symptom, such as flicks of the tail within the egg, when we cannot see how the fish would grow up and succeed under natural conditions – in short: are early behavior alterations relevant for the fitness of the animal and ultimately the population? Potential weaknesses of the embryonic behavior approach can be imagined in both directions: abstract early responses may be overly sensitive, as pointed out for enzyme assays above, or altogether miss toxic effects that only manifest themselves in behaviors that develop later in life (feeding or reproductive behaviors come to mind). As the underlying motivation remains to define a sufficiently powerful alternative test battery so that only a minimum of *in vivo* experiments is required, similar to the genotoxicity battery of ToxBox (Grummt et al., 2018b), those that do need to be carried out should possess a high level of confidence.

To begin to grasp which behaviors might be suitable for the intended purpose of inferring population relevant behaviors from more abstract early patterns, a brief exploration of the multitude of paradigms (set of conditions allowing the observation of a specific behavior) that have been developed for zebrafish is certainly helpful.

3.5 The role of behavior in ecotoxicity testing

Assessment of behavioral alterations under substance exposure has mostly played a role in pharmacological studies so far. While there is a wealth of thoroughly documented and sometimes standardized paradigms, it is important to keep in mind that the questions posed from an ecotoxicological standpoint may differ so significantly that methodic adjustments are required. For instance, those studies are routinely interested in the "rescue" of individual fish with acute treatments against a prevailing (often genetic) damage while ecotoxicology is ultimately concerned with the reaction of entire systems of wild-type organisms against prolonged exposure from an early age. Isolating natively shoaling fish for ease of observation, while certainly reasonable in many toxicological studies, may cause a major deviation from the natural-like behavior I am interested in as a baseline (Bass and Gerlai, 2008, Bencan et al., 2009). However, the underlying behaviors of many pharmacological paradigms do not only illuminate disease-like behaviors but can confidently be expected to play a role in predation statistics and thereby population fitness in the wild.

3.5.1 Testing for changes in adult fish behavior

As briefly mentioned before, apical population relevant behaviors are most directly observable in adult fish. While such experiments are ideally avoided for ethical reasons, they are what new approaches ultimately need to be compared with and shall therefore be outlined in the following.

To analyze behavior in the presence of danger, the most direct approach is to confront the experimental fish with one of its predators. The strongest response would be expected if both species are within the same tank and indeed this has been attempted (Barcellos et al., 2007, Nair et al., 2017), but of course the loss of some fish is to be expected with this approach and the predator may show considerably varying aggression behavior depending on its reduced appetite during later trials or its own handling-induced stress level. Putting the predator behind a transparent divider or into a neighboring tank eliminates the risk of actual predation, but the predator may lose interest over time and generally still behaves unpredictably over time (Bass and Gerlai, 2008, Luca and Gerlai, 2012, Stewart et al., 2014a). Several authors have successfully utilized animated images of a predatory fish or even certain abstract shapes on screens outside the tank to simulate the threat, which solves the aforementioned problems (Ahmed et al., 2012, Gerlai et al., 2009, Luca and Gerlai, 2012). One shortcoming that remains, however, is the limitation to visual cues whereas fish also rely on their lateral line organ to detect movement through fluctuations in water pressure, especially if one of these two partially redundant senses may be affected by a toxin. A moving stimulus within the tank would therefore be an interesting further refinement (Cianca et al., 2013, Ladu et al., 2015).

Whichever of these stimuli is used, the expected responses from the experimental fish would be increasing the distance between themselves and the threat and – if multiple fish are assessed together, which has been the exception so far – huddle in a tighter shoal for protection (Bass and Gerlai, 2008, Stewart et al., 2014a).

The natural tendency of many fish, including zebrafish, to shoal with conspecifics is also the basis of an independent paradigm. In "social preference", "group preference" or simply "shoaling" tests, an isolated fish is placed in a wide tank and its preference for the side where other fish are visible (by means of a screen or a second compartment with life fish) *versus* the opposite, empty side is evaluated (Blaser and Gerlai, 2006). Effects disrupting normal shoaling behaviors can be interpreted as decreasing the relatively isolated fish's chance of survival when facing a predator as safety lies in the masses.

Less directly related to predation, but usually postulated to originate from anti-predatory behaviors, are anxiety responses. The rationale is that there must be a healthy balance between caution and exploration which keeps an animal safe while allowing opportunities to feed and mate even in potentially dangerous areas. Deviations from this baseline are relevant in both directions: individuals that are overly bold are at a higher risk of predation, but those too anxious to come out of hiding, so to speak, may become malnourished and less proliferative as a result. Anxiety-based paradigms can be used to compare a treated group's response in a stress or conflict situation with the presumably normal baseline behavior of an untreated group. The earlier case if employed in novel tank tests, where fish are observed immediately after being placed into a foreign environment. The common response to this is bottom dwelling (or rather, escape from the surface) for 2-3 minutes followed by gradual exploration of the entire tank until habituation is achieved (Cachat et al., 2011, Levin et al., 2007, Maximino et al., 2012). The apparent bottom dwelling is also called "diving response" or "geotaxis" by different authors (e.g. Maximino et al., 2013a, Sledge et al., 2011, Stewart et al., 2012). Almost all published studies have only applied the novel tank paradigm to isolated adult fish (e.g. Egan et al., 2009, Kulkarni et al., 2014, Orozco-Hernandez et al., 2022, Wong et al., 2010).

Rather than such a gradual change in behavior, a clear conflict between two choices may be beneficial for some hypotheses and can be achieved in light/dark preference tanks (Maximino et al., 2010). The walls and bottom of these elongated tanks are colored white in one and black in the other half and fish can be observed from above – since zebrafish and many other species have darkly pigmented backs, they intuitively prefer the black portion of the tank where they are hidden from predators above (Maximino et al., 2010, Maximino et al., 2012).

I shall not go into detail about even more complex behaviors here as my goal lies in the opposite direction: which of these behaviors may already be present in earlier life stages at a fundamental level?

3.5.2 Larval and embryonic alternatives to behavior assays in adult fish

The earliest observable behavior pattern in zebrafish embryos is the onset of spontaneous tail motion within the egg, or "coiling", around 17 hours post fertilization (hpf; Brustein et al., 2003). The time of onset, duration or frequency of these tail coils may be altered by external factors including toxins (Zindler et al., 2019a, Zindler et al., 2019b). In addition to spontaneous coiling, stimuli physical manipulation of the container (tapping) or transitions between light and darkness (photomotor response) can be utilized to evoke bursts of activity in healthy embryos and exposure-induced alterations thereof may be detected easily and reliably (Kokel et al., 2010, Kokel and Peterson, 2011).

Later in embryonic development, touch response and burst swimming behavior gradually develop. Whereas coiling originates from a set of electrically linked neurons, these are already based on more mature neural interactions through chemical synapses (Brustein et al., 2003). If treated embryos show an accelerated touch response, it is argued their predator avoidance has also been increased (Qiang et al., 2016). Swimming, "motility", or "locomotor" behavior may be observed in terms of baseline activity with and without treatment or the light may additionally be switched off after the first half of the trial (Richards et al., 2008, Selderslaghs et al., 2010, Winter et al., 2008). Interestingly, activity in the darkness usually increases: contrary to their later preference for dark areas, the so far unpigmented larvae prefer bright environments at this stage and presumably sense danger in a sudden onset of darkness as if it were a menacing shadow from above (Maximino et al., 2010). Larvae around 6 dpf have been shown to be dramatically more active than one or two days earlier and are thus commonly used for locomotor activity assays (Mora-Zamorano et al., 2016, Padilla et al., 2011, Selderslaghs et al., 2010). Video recording and automated tracking tools usually play an essential role in calculating the movement parameters for each larva in a multi-well test setup (Colwill and Creton, 2011).

The problem with early behaviors is that they are often difficult to interpret. For instance, reduced tail coiling activity inside the egg may be an early indication of motor or sensory impairments later in life, but an affected embryo might just as well develop into a healthy animal (perhaps with a few hours of delay that will not be relevant for its adult life). Therefore, this dissertation aims to put such early alterations into perspective by comparing them to behavioral effects of prolonged exposure to the same substances. Best possible consideration of natural conditions in my behavior assays shall help to evaluate population relevance in order to answer the fundamental question for a feasible strategy of identifying neurotoxins without adult fish testing.

4. Recurring methods

This section includes content from a manuscript which was originally written by me and served as the basis for the following joint publication:

> **Lukas Frese & Thomas Braunbeck (2022):** Adapting classic paradigms to analyze alterations of shoal-wide behavior in early-life stages of zebrafish (*Danio rerio*) – A case study with fluoxetine. Neurotox Teratol 95, 107136. https://doi.org/10.1016/j.ntt.2022.107136

Reprinted with permission from Neurotoxicology & Teratology 2022. Copyright © 2022 Elsevier Inc.

4.1 Fish maintenance: stock population & husbandry

Adult wild-type zebrafish (*Danio rerio*) of the Westaquarium strain were obtained from the breeding facilities at the Aquatic Ecology and Toxicology Group at the Center for Organismal Studies (University of Heidelberg; licensed under no. 35-9185.64/BH). Fish maintenance, breeding conditions and egg production in these facilities were described in detail by Lammer (2009). In brief, the stock population was kept in large glass tanks at a density ≤ 1 adult fish per liter. Constant flow of fresh water, internal mechanical filtration and regular chemical tests ensured good water quality. The environment was enriched by small quantities of free-floating filamentous algae while all walls and floors were scraped weekly to prevent formation of biofilms.

Breeding groups of up to 20 individuals were assembled from non-related males and females between 6 and 24 months of age. These were kept in bare tanks without filtration or other possible interference factors. Instead, water quality was ensured by a relative high flow of fresh water compared to the tanks' volume. Other than that, the holding conditions were the same as for the stock population.

Fluorescent light tubes at the room ceilings and above each tank supplied a 14:10 h light/dark cycle with the first and last 30 minutes of each "day" characterized by decreased direct light intensity to simulate dawn or dusk, respectively. Water (hardness approx. 2.5 mmol/L, pH 8.0 ± 0.2, NO_3 < 10 mg/L, NO_2/NH_4 not detectable) was well-oxygenated throughout the system by aeration stones in all mixing, storage, and fish housing tanks and kept at 25.5 ± 1.0 °C by thermostat-controlled heating mats underneath the tanks. All adult fish were fed twice daily; once with freshly hatched *Artemia* nauplii (48 h incubation; Sanders, Ogden, Utah, USA) and once with dry flake food (TetraMin™, Tetra, Melle, Germany). Excess food and feces were extracted daily.

To facilitate spawning, up to four parallel breeding groups were selected the afternoon before the intended harvest and transferred to separate spawning units consisting of mesh-bottom tanks submerged in shallow water. These tanks were slightly angled to promote "beaching" behavior of females laying eggs and outfitted with artificial grass as an additional stimulus. The mesh allowed eggs to fall into collection dishes below but held back the adult fish, effectively preventing filial cannibalism. Within an hour after spawning at dawn, the adult fish were

returned to their respective holding tanks and the eggs were collected and cleaned with fresh water before further use.

4.2 Test chemicals

Unless stated otherwise, all chemicals used were purchased from Merck (Darmstadt, Germany) at a minimum purity of 98.0 %. Fluoxetine hydrochloride (FLX; CAS no. 56296-78-7) and carbamazepine (CBZ; CAS no. 298-46-4) were purchased as Pharmaceutical Secondary Standards. Paraoxon-methyl (PXM; CAS no. 950-35-6) and tris(1,3-dichloroisopropyl) phosphate (TDCPP; CAS no. 13674-87-89 were purchased as PESTANAL® analytical standards.

In order to avoid systemic effects and to only record specific neurotoxic effects not masked by general toxicity (Grummt et al., 2018b, Stengel et al., 2017a), the maximum exposure concentrations were based on ≥ 96 h EC_{10} values from the literature or my own preliminary experiments based on fish embryo tests (OECD TG 236; (OECD, 2013b), which were partly extended to up to 12 dpf. The EC_{10} has also been termed highest non-teratogenic concentration (HNTC) in this context (Selderslaghs et al., 2010).

5. Development of a shoal-wide juvenile behavior assay with fluoxetine as a model substance

This chapter includes content from a manuscript which was originally written by me and served as the basis for the following joint publication:

> **Lukas Frese & Thomas Braunbeck (2022):** Adapting classic paradigms to analyze alterations of shoal-wide behavior in early-life stages of zebrafish (*Danio rerio*) – A case study with fluoxetine. Neurotox Teratol 95, 107136. https://doi.org/10.1016/j.ntt.2022.107136

Reprinted with permission from Neurotoxicology & Teratology 2022. Copyright © 2022 Elsevier Inc.

5.1 Introduction

The long-lasting increase in the numbers and amounts of anthropogenic chemicals has inevitably led to the accumulation of chemicals with potentially deleterious effects on organisms, populations and ecosystems. Although large-scale disasters such as the thalidomide scandal hopefully belong to past decades (Franks et al., 2004, Paine, 2017, Vargesson, 2015, 2019) and environmental toxicity testing has led to restrictions or complete bans on the production of the most dangerous chemicals, there is still a long list of scary discoveries of long-term low-exposure effects (Grandjean and Landrigan, 2006, Jamal et al., 2002). Neurotoxins are a particularly worrying subset of bioactive anthropogenic substances, often causing subtle developmental damage that may remain undetected at an individual level, but has the potential to negatively affect entire populations (Claudio et al., 2000, Stein et al., 2002). Nevertheless, less than 1 % of all chemicals sold on global markets have been estimated to be evaluated against their potential neurotoxic and/or neuro-modulating properties (Grandjean and Landrigan, 2006, Legradi et al., 2018, Wlodkowic et al., 2022).

As a consequence, there is growing concern about the relevance of neurotoxicants for, e.g., the water cycle, which has led to specific projects such as ToxBox (Eckhardt et al., 2017, Grummt et al., 2013) and its follow-up project NeuroBox (Grummt et al., 2018a, 2020, Kuckelkorn et al., 2020), which were, among other purposes, designed to improve current protocols for neurotoxicity testing and to optimize neurotoxicity testing strategies. New test protocols should be simple, avoid animal testing (at best, be *in vitro*) and allow for high-throughput assessment to keep pace with the speed of chemical innovation. Yet, since *in vitro* screening tools inherently bear the risk of (over-)simplification, there is also a need for tools for bridging *in vitro* observations to the real world of complex organisms. Since, at least in the European Union, zebrafish (*Danio rerio*) embryos are regarded not protected until the age of 120 h (Strähle et al., 2012), they not only provide a promising model system in ecotoxicity testing (Braunbeck et al., 2015, Embry et al., 2010, Halder et al., 2010), but also serve as a compromise between *in vitro* screening and conventional *in vivo* ecotoxicity testing (Braunbeck et al., 2015).

Even though early life-stage toxicity only represents a developmental "snapshot" for the identification of long-term toxicity, developmental stages have been identified for many

chemicals as the most sensitive stages in the life cycles of fish (McKim, 1977). Thus, a test system that reliably detects *whether or not* a chemical bears a risk of neurotoxicity might be an important step forward to provide authorities with data to initiate measures to prevent harm in accordance with, e.g., the concept of the Health Orientation Value (HOV; Grummt et al., 2018b, UBA, 2003).

As mentioned earlier, the zebrafish (*Danio rerio*) has received increasing interest in general toxicology and ecotoxicology over more than 20 years for multiple reasons (Braunbeck, 2009, Braunbeck et al., 2015, Busch et al., 2011, de Esch et al., 2012, Di Paolo et al., 2015, He et al., 2014, Lammer et al., 2009, Parng et al., 2002, Scholz et al., 2008, Simeon et al., 2021, Stegeman et al., 2010, Weigt et al., 2011). Since many neurological structures and processes are conserved across vertebrates, however, zebrafish have also been developed as a general model for neurodevelopmental disorders and neurotoxicity (Best and Alderton, 2008, Kanungo et al., 2014, Spitsbergen and Kent, 2003, Ton et al., 2006, Tropepe and Sive, 2003, Von Hellfeld et al., 2022), and zebrafish have also increasingly been used in traditionally rodent-dominated pharmacological behavior studies (Brotzmann et al., 2021, Kalueff et al., 2014, Kysil et al., 2017, Stewart et al., 2014a).

Apart from classical markers of neurotoxicity such as acetylcholine esterase (Behra et al., 2004, Kais et al., 2015, Tilton et al., 2011) or morphological alterations in central sensory organs (Stengel et al., 2017a, Stengel et al., 2017b, Wlodkowic et al., 2022), most neurotoxicological studies in zebrafish used changes in behavior as endpoints of neurotoxicity (e.g., Colwill and Creton, 2011, Dishaw et al., 2014a, Richendrfer et al., 2014). Based on technical advances, e.g. the ability to track fish in a 3D environment, an extended range of endpoints has also become possible in behavior testing (Cachat et al., 2011); however, most established paradigms rely on a single adult fish as test subject, which is certainly not ideal for ecotoxicological purposes. First of all, in nature, many fish species including zebrafish form shoals and suffer from measurable stress when isolated (Pagnussat et al., 2013, Parker et al., 2012). Second, although there are some examples of substances that continue to affect adult fish after a short-term exposure at a precise point in development (Andersen et al., 2000, Truong et al., 2012), chronic and sub-chronic exposures from an early age certainly represent more realistic environmental scenarios and will usually reveal different – often much lower – effect concentrations (Ansai et al., 2016, Gaworecki and Klaine, 2008, Pelli and Connaughton, 2015). However, long-term exposure from early development to the adult stage is not only prone to technical failure and very cost-intensive, but is also in conflict with animal welfare considerations.

Therefore, an extended early-life exposure scenario such as that used by OECD TG 210 (OECD, 2013a) is likely to represent a feasible and population-relevant exposure scenario. In accordance with the mission to reduce the number of animals used for toxicity testing (Hutchinson et al., 2016, Rawlings et al., 2019, Vaughan and van Egmond, 2010) and to keep additional fish toxicity testing economic (Oris et al., 2012, Rawlings et al., 2019, Rufli and Springer, 2011), existing test guidelines can be expanded to cover additional behavior endpoints rather than to introduce entirely new methods. To this end, I conceived a method to complement OECD TG 210 by behavioral endpoints recorded in groups of free-swimming juvenile fish: a novel tank test and a predator response assay. Given its well described anxiolytic effect in novel tank tests (Cachat et al., 2011, Egan et al., 2009, Wong et al., 2010), the selective serotonin

reuptake inhibitor (SSRI) fluoxetine was selected for this proof-of-concept study. Its efficacy is based on the prolonged stimulation of postsynaptic neurons *via* increased serotonin levels in the synaptic cleft (Parolini et al., 2019, Wong et al., 1974). As a drug widely prescribed as an antidepressant, fluoxetine has continuously been discharged into the environment (Stewart et al., 2014b, Tisler et al., 2019, Zindler et al., 2019b, 2020b, 2020a), where it is not readily photolyzed or microbially degraded, but rapidly adsorbs to sediments (Kwon and Armbrust, 2006).

The novel tank test, also known as "novel open tank task" or "novel tank diving test" (Blaser and Gerlai, 2006, Rosemberg et al., 2011), records reactions of fish to an unknown environment. Typically, zebrafish will show a strong initial preference for the bottom of the tank, before they gradually explore the entire available volume (Levin et al., 2007). The diving response and temporary bottom-dwelling are interpreted as an anti-predatory behavior, i.e., small fish are thought to instinctively avoid danger from above by moving away from the surface in potentially dangerous situations (Gerlai et al., 2000, Kysil et al., 2017, Levin et al., 2007, Sackerman et al., 2010). Thus, the rate of diving and subsequent habituation allows conclusions about the "anxiety status". Based on the assumption that a certain level of anxiety exists in any situation that balances an individual's contradicting motivations to avoid danger and to explore and forage (Maximino et al., 2010), anxiolytic or anxiogenic effects are likely to reduce life expectancy and/or nutritional state, respectively, both impairing individual fitness and eventually population-relevant performance (Dzieweczynski and Hebert, 2012, Pelli and Connaughton, 2015).

There is no standard protocol for novel tank tests so far, but various conventions have largely been accepted: A suitable test tank should have a different shape than the regular "home" tank to strengthen a novelty response. This has frequently been achieved by a particularly narrow test tank (Bencan et al., 2009), and two horizontal depth zones ("top" and "bottom") have proven sufficient and widely replaced earlier, less labor-efficient three-zone protocols (Egan et al., 2009, Cachat et al., 2010). The trials can be kept rather short, since habituation may be expected within 6 minutes (Wong et al., 2010). The method is usually applied to individual adult zebrafish and has been declared unsuitable for larval zebrafish (Cachat et al., 2010).

Whereas the novel tank test identifies only less specific anxiety responses, stress can also be induced directly by confronting the test subjects with a live predator or key stimuli thereof in a predator response assay. Fish are known to show a variety of responses in such a situation, ranging from freezing and hiding to active escape behavior (Ahmed et al., 2011). An intuitive and much utilized metric is the distance between the fish and the location of the stimulus, which is expected to increase upon presentation of a predator (Ahmed et al., 2011, Bass and Gerlai, 2008). More subtle, but also ecologically important, are changes in shoaling behavior: To avoid predation, zebrafish will form tighter shoals when stressed (Bass and Gerlai, 2008, Miller and Gerlai, 2007, Stewart et al., 2014a). Assays focused on single fish may instead include measurements of erratic swimming movements and "jumps", isolated bursts resulting from powerful tail fin strokes (Bass and Gerlai, 2008, Gerlai, 2010) meant to increase the chance to escape from a potential predator.

Since confrontation with a live predator is likely to result in the death of at least some test fish, predator response assays are usually not conducted with real predators; rather, prey and

predators are placed in adjacent tanks or dummies are used to simulate the danger. The usual way to make safe use of a live predator is to place it in a separate tank next to that of the test subjects, optically concealable *via* opaque sheets, if needed. Unfortunately, olfactory or lateral line perception cannot contribute to sensing the predator in such a setup, and the quality of the optical cues varies depending on the predator's own behavior (Bass and Gerlai, 2008). Computer screens can replace the live predator to eliminate the latter problem (Ahmed et al., 2011, 2012, Luca and Gerlai, 2012), but also exclusively deliver optical stimuli. Interestingly, certain abstract patterns like a moving dot have been shown to elicit even stronger responses than the most stress-inducing sympatric predator, the Gangeatic leaffish *Nandus nandus* (Ahmed et al., 2012, Luca and Gerlai, 2012). Despite the advantages of screen images, I chose a simple "robotic" predator model within the same tank to stimulate both optical and lateral line sensing. Movements were kept small to not obstruct the camera's field of view.

Although the aforementioned tests have usually been conducted with single adult fish – except for one shoaling assay conducted with up to five individuals (Bass and Gerlai, 2008) –, the present study was designed to analyze options to conduct both novel tank and predator response tests with groups of juvenile or even larval zebrafish for the following reasons: In stagnant waters, zebrafish have been shown to prefer larger shoals from an early age (Buske and Gerlai, 2011) with an estimated natural shoal size of about 10 individuals (Suriyampola et al., 2016). While single fish are easier to observe (especially automatically), isolation of naturally shoaling fish inevitably induces additional anxiety interfering with the treatment (Bass and Gerlai, 2008, Gerlai et al., 2000). Especially acute exposure studies with an individual pre-treatment phase in small beakers are likely to trigger an unrealistic baseline behavior that might compromise the potential to predict population-relevant effects. Since reciprocal enforcement of risk perception might make groups more sensitive in settings such as the predator-response test (Giacomini et al., 2015), there is also potential to reveal effects at concentrations previously unnoted.

5.2 Materials and methods

5.2.1 Egg collection and rearing

Zebrafish (Danio rerio) eggs for experimental use were obtained from genetically heterogenic spawning groups as described in OECD TG 236 (OECD, 2013b) and allowed to hatch in 1.7 L polycarbonate tanks (Tecniplast, Italy) within a 26 °C incubator (Binder, Tuttlingen, Germany). Large batches were split among several such tanks so that each held less than 300 eggs, and water was exchanged daily to ensure good quality. From the 5th day post-fertilization (dpf), larvae were fed *ad libitum* with *Paramecium caudatum* (own culture) twice daily. At 6 dpf, larvae were transferred to 21.5 L full-glass rearing aquaria (18 × 40 × 30 cm) supplied with a continuous 1× flow-through water replacement and gradually received larger food particles: from day 9 to 11, larvae were fed with *Paramecium*, Nobil fluid 'Artemia' (JBL, Neuhofen, Germany) and Micron powder (Sera, Heinsberg, Germany). From day 12 to 15, they were given *Artemia* nauplii in addition to *Paramecium*, Nobil fluid and Micron powder, before *Paramecium* was removed from their diet from day 16. All steps were conducted under the same water quality and light regimes as described in the beginning.

5.2.2 Exposure to fluoxetine

The EC_{10} value for 96 h FETs with fluoxetine was found to be 7.39 mg/L (Zindler et al., 2019b). Since preliminary range-finding experiments over 12 d revealed a decline of EC_{10} values to 80 µg/L (cf. Airhart et al., 2007), the final concentrations range for my experiments was 0, 5, 10, 20 and 40 µg/L fluoxetine, which also filled a criticized gap in the knowledge of fluoxetine dose-response relationships in aquatic organisms (Sumpter et al., 2014).

Embryos and larvae were exposed in 5 L flow-through glass exposure tanks (18 × 40 × 7 cm), which were maintained at 26 ± 1 °C and had been preconditioned with the respective test concentrations for 24 h as to saturate any potential binding sites along the silicone seals. For exposure, a 2× exchange (10 L/d) of the test solutions was maintained under continuous flow-through conditions according to OECD TG 210 (OECD, 2013a) using Minipuls 3™ peristaltic pumps (Gilson, Limburg, Germany) for the test solutions and rotameters (Rota Yokogawa, Wehr, Germany) for the dilution water (Fig. 2). Water and test solution flow rates were monitored on a daily basis.

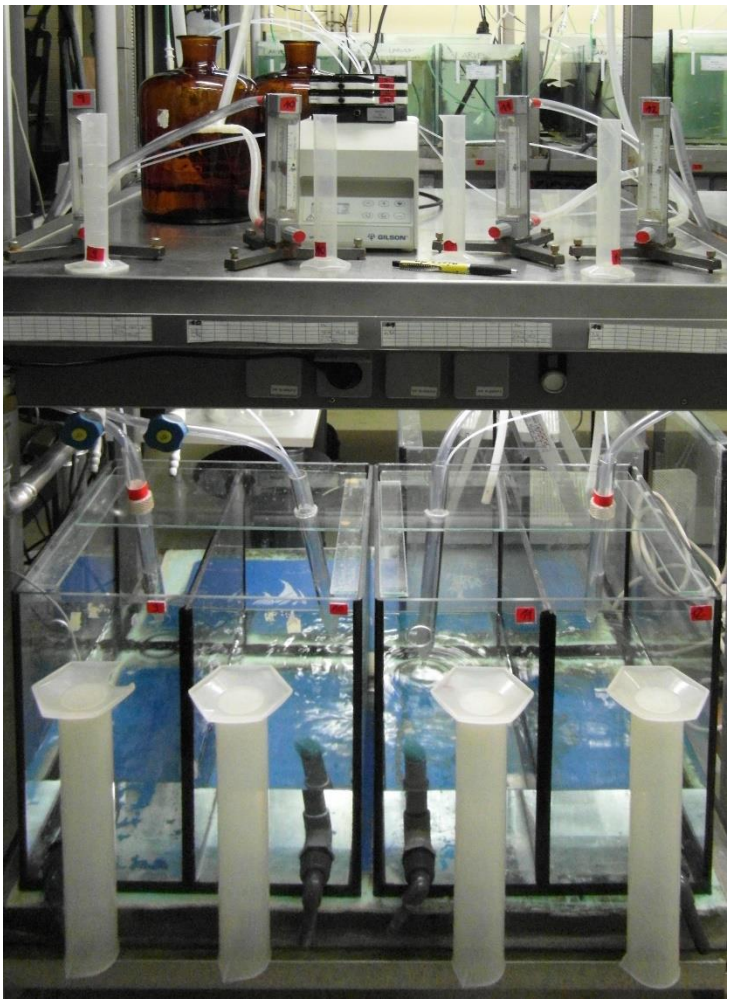

Fig. 2: Flow-through exposure setup in the fish facility. Fresh water flows down from tanks near the ceiling (not shown) through thick silicone tubes and rotameters that regulate how much reaches the tanks on the bottom rack. In parallel, thin Teflon tubes are used to pump precise amounts of the appropriate chemical stock solution from brown glass bottles (top, left) into each exposure tank by means of a peristaltic pump (top, center).

At the onset of exposure (21 dpf), 10 larvae of undetermined sex were transferred to the exposure tanks for each test concentration and a dilution water (negative) control. To avoid air or net contact of these fragile developmental stages, fish transfer was accomplished by 3 ml plastic pipettes with enlarged openings and small glass dishes. Except for the delayed onset, exposure was carried out according to OECD TG 210 (OECD, 2013a) with the following modifications: In order to minimize stress by cleaning, feces and excess food were not removed before the end of exposure. To minimize accumulation of feces and food particles, the amount of food given was controlled carefully. In combination with flow-through conditions, good water quality could be guaranteed, as could be confirmed by continuous monitoring of pH as well as nitrate, nitrite and ammonia (low detection limits of titration). Since this study was designed as a proof-of-concept and did not aim to determine exact effect concentrations (e.g., EC_{10}), the number of fish per test concentration were reduced to 10 instead of 20 as required according to OECD TG 210 (four replicates per concentration). Fish were exposed for 14 d (21 - 35 dpf) with daily observation of the fish. Thus, moribund individuals (if any) could be removed swiftly.

5.2.3 Observation tank

For video recordings, an 8 L glass tank (25.7 × 18 × 17.3 cm) was used (Fig. 3). Although a narrower tank with “up” and “down” as the main possible swimming directions would have made observations easier, it was deemed important for natural behavior that the fish should move at least as freely under observation as in their rearing tanks. Therefore, a tank with an even larger volume than what the fish were accustomed to was selected. To eliminate any effect from alarm pheromone residues from previous experiments, the tank was filled with fresh water from an unused rearing tank in the same room (26 ± 1 °C) prior to the introduction of each new experimental group. “YourLED” light strips (daylight: 6000 K; Paulmann, Springe, Germany) were mounted on custom-angled holders along the upper edges of the tank for optimal light coverage.

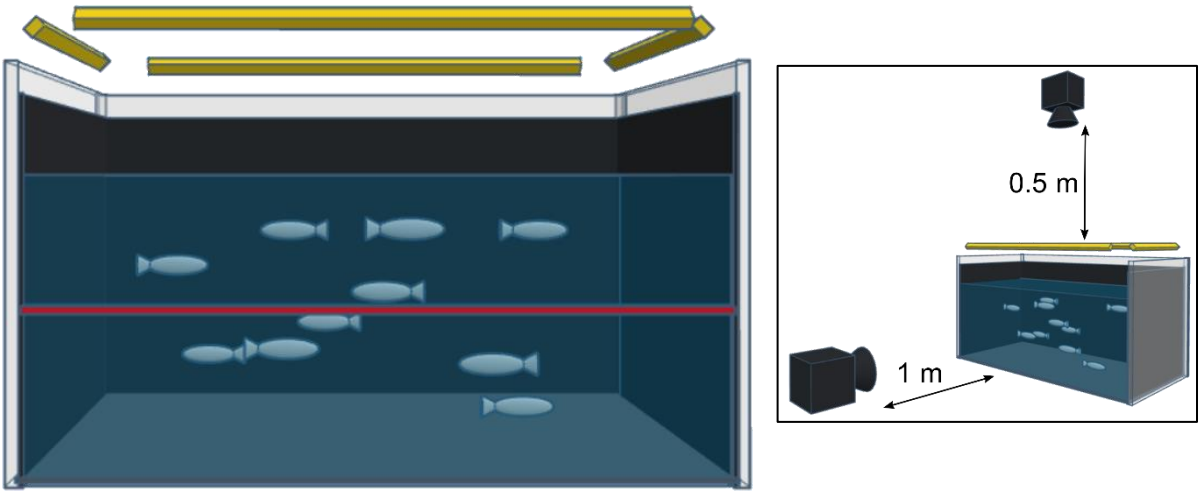

Fig. 3: Schematic view from the front camera angle with the virtual line (red) separating the upper and bottom halves of the tank. LED strips (yellow) illuminate the tank from above. The cameras are mounted too far from the tank to be shown to scale (box).

By means of an Arduino UNO microcontroller (Arduino, Turin, Italy) and a simple switching circuit, the brightness of each light strip was adjusted individually to minimize formation of shadows or overly bright areas. The bottom, rear and side walls were coated with matte black PVC films ("d-c-fix", Hornschuch, Weißbach, Germany) to avoid reflections and optimize contrast to the fish.

Two acA1300-60gm cameras (Basler, Ahrensburg, Germany) were installed above the tank ("top") and in front of the open wide side ("front"; Fig. 3). An LMVZ4411 lens (Kowa Optical Germany, Düsseldorf, Germany) was mounted on each camera. Cameras were set up at the furthest possible distance and with barely opened apertures to achieve an increased depth of field. The camera lenses were shielded by black sheets of cardboard to minimize reflections in the uncovered glass wall or the water surface. To avoid reflections in the water surface, the front camera was positioned at a height and angle that made the water surface appear as merely a line (Fig. 3). Ideal frame acquisition parameters were determined by means of the Basler pylon Viewer software (v. 5.0.11.10913; Basler, Ahrensburg, Germany) and kept constant for all replicate experiments. Video streams were also captured using pylon Viewer. Digital enhancement was avoided, and images from the "front" camera were saved as lossless TIFF files, since these were expected to be the essential source of data. The top stream was recorded as a single AVI video. The framerate was reduced to 10 fps to make sure that the available recording bandwidth was not exceeded when running both cameras in parallel.

5.2.4 Behavior testing

Behavior tests were carried out at 35 dpf, i.e. after 14 days of exposure to fluoxetine. The sequence of test groups was randomized. All tests were carried out between 10 am and 3 pm in agreement with the timeframe recommended by Rosemberg et al. (2011) to avoid artifacts due to the naturally increased nighttime boldness (Maximino et al., 2010, Rosemberg et al., 2011). All fish were experimentally naïve and the order in which the different treatment groups were observed was randomized. Video recording was started immediately prior to transferring the entire shoal from the exposure tank to the observation tank.

The first six minutes of every test were recorded as a novel tank test without any further manipulation. This was followed by the introduction of a 3D printed model of the Gangeatic leaffish (*Nandus nandus*, Fig. 4) to record variations in predator response behavior.

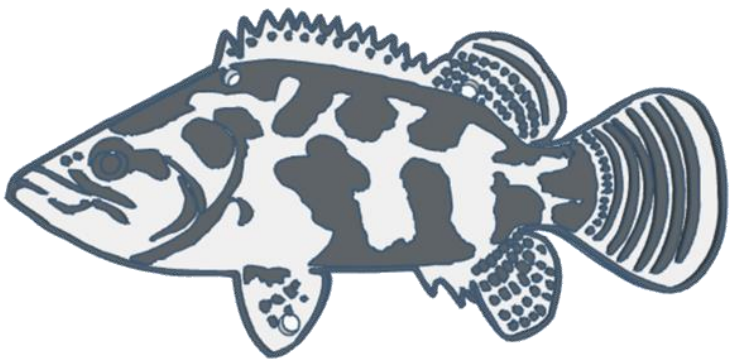

Fig. 4: CAD model of the predator dummy. The leaffish was 3D printed with black and white plastic for maximum contrast and hung from transparent threads through the holes at the top.

The predator dummy was moved semi-randomly *via* transparent threads by a small motor located outside the tank, which was driven by the same microcontroller that operated the light strips. After recording a sequence of novel tank and predator response tests, the shoal was euthanized by submersion in a 400 mg/L tricaine mesylate (MS-222) solution on ice and fixed in modified Davidson's fixative (Latendresse et al., 2002).

After each test sequence, the water in the observation tank was replaced completely before the introduction of the next group to remove olfactory cues. Especially the cyprinids' "alarm pheromone", which may be released by a fish brushing strongly against the net, would otherwise cause increased stress and avoidance behavior in the following individuals. As an unusually sterile environment might also be disturbing for the fish, water from a long-running tank closely resembling the rearing aquaria (except for the absence of fish) was used.

5.2.5 Video analysis

Objective analysis was made possible by an area-of-interest template that was digitally laid over the image sequence with the freeware GhostIt! V. 1.04 (Pandina, 2002) and was not changed between replicates. Depending on the behavior test, this graphic consisted of the observation tank's outline and (a) a horizontal line separating the tank into a top and bottom half for novel tank tests (Fig. 1), or (b) three evenly spaced vertical lines separating the tank into four zones of decreasing distance from the predator dummy at the far right (Fig. 5).

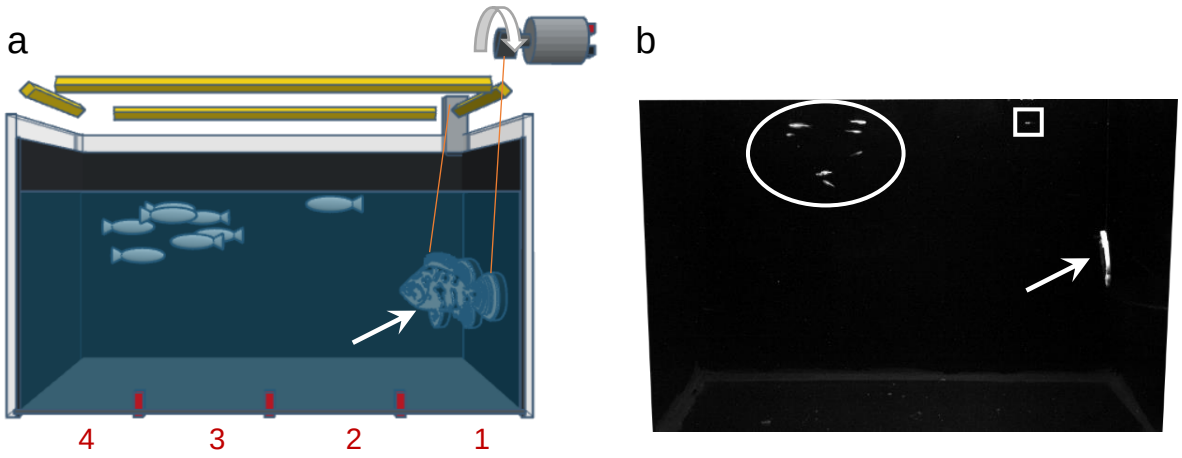

Fig. 5: Principle of the predator response assays. a) Schematic setup with a plastic dummy (arrow) held by strings that are plucked by a motor above the tank. b) Still frame recorded during a predator response test. The 3D printed dummy has been lowered into the tank and is partially visible on the right (arrow; not facing the camera). The majority of zebrafish keep a distance and have formed a tight shoal (circle) while a single fish is much closer to the dummy (box).

Then I would record the number of individuals in each zone for each timepoint. Fish touching or crossing a line were attributed to the zone containing the larger part of their visible body area; if this could not be determined with certainty, both zones were counted to contain 0.5 fish.

When two or more fish would overlap and appear as one, the neighboring frames and the top perspective were used to distinguish between them to make sure all individual positions were counted.

Every tenth frame (1 per second) of the recorded material was analyzed in such detail, while the remaining frames only served as a reservoir to clarify unclear swim paths by providing the necessary context. The start of a novel tank test was defined as the first frame after the emptied net had completely left the field of view of the front camera. The predator response tests began with the complete introduction of the predator dummy into the water. From these starting points, both types of videos were assessed for 240 representative frames (four minutes).

5.2.6 Data analysis

In order to demonstrate intra- as well as inter-trial variability, data were examined both as collected (n = number of frames, more realistic variance) and pooled per trial (n = number of replicates, part of the variance masked by single means). The number of individual fish in each zone at any given second was used to calculate different behavior scores relating to the following hypotheses (Table 1): neurotoxic substances may alter (a) the shoal's diving and subsequent habilitation behavior in a novel tank test and (b) its average distance from a perceived predator as well as the strategy of cohering more strongly when confronted with such a danger.

Table 1: Calculation of behavior endpoints in the novel tank and predator response tests. Novel tank endpoints focus on changes in the delay to reaching the potentially more dangerous upper half of the observation tank. The predator response scores quantify observations of flight reactions (distance to the predator) and shoal coherence as an alternative defensive strategy.

Test design	Score title	Score calculation
Novel tank test	Upper half preference (UHP)	$\frac{no.of\ fish\ (top\ half)}{no.\ of\ fish\ (total)}$
	Above-control UHP (per minute)	$\frac{no.of\ UHP\ scores\ (treatment) > avg.UHP\ (control)}{60}$
Predator response test	Predator distance	$\frac{4 \times no.fish\ (furthest) + 3 \times \ldots + 1 \times no.of\ fish\ (closest)}{total\ no.\ of\ fish}$
	Shoal coherence	$\frac{no.of\ zones\ containing\ fish}{no.of\ fish}$

Comparisons among behavioral scores from different trials and treatments were carried out by Kruskal-Wallis ANOVA-on-ranks and Dunn's test as *post hoc* using SigmaPlot 14.0 (Systat Jandel, Erkrath; Inpixon, Düsseldorf, Germany). All results from chemical treatments were compared pairwise to those from negative controls.

5.3 Results

5.3.1 Fluoxetine heavily diminished the novelty response

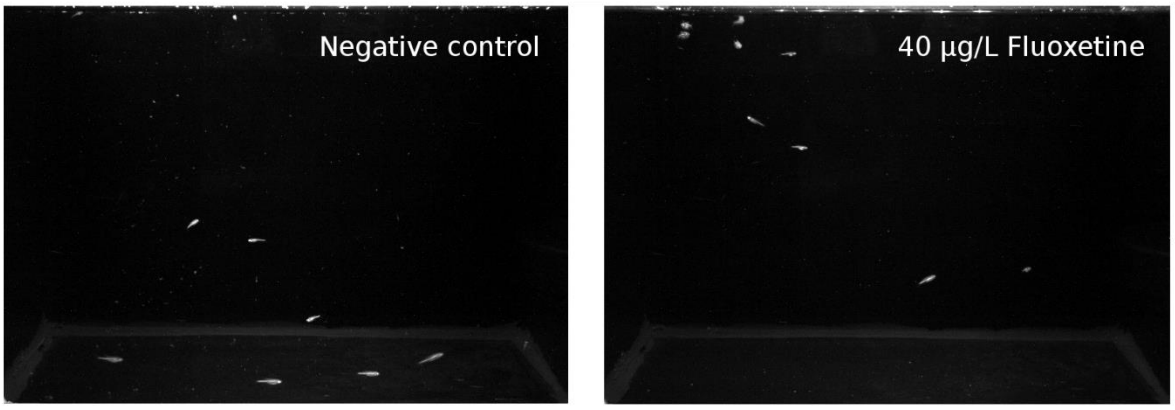

Fig. 6: Typical reaction of 34 d old zebrafish (*Danio rerio*) larvae in the novel tank test: Approx. 1 min after transfer to the novel tank, control larvae show a strong initial preference for the bottom of the tank as part of an anti-predatory behavior (left), whereas larvae previously exposed to 40 µg/L fluoxetine already start exploring the upper sections of the tank, thus indicating a reduction in their "anxiety status" (right).

In the novel tank tests, effects of 20 and 40 µg/L fluoxetine were so conspicuous that they were visible to the naked eye (Fig. 6): The majority of fish swam close to the surface for the main part of the tests; although they were still capable of diving, they only did so infrequently and seemingly independent from the time spent in the novel tank. In contrast, control fish consistently immediately dove to the bottom of the tank as soon as they could leave the net. Usually, control fish stayed at the tank bottom for 2 - 3 min before exploring the upper half volume. Given the clear segregation of fluoxetine-exposed fish into different areas of the tanks, the portion of individuals located in the upper half tank was computed as "upper half preference" (UHP). A comparison between the 10-sec UHP means of controls and 40 µg/L fluoxetine clearly revealed a highly significant increase in UHP (Fig. 7).

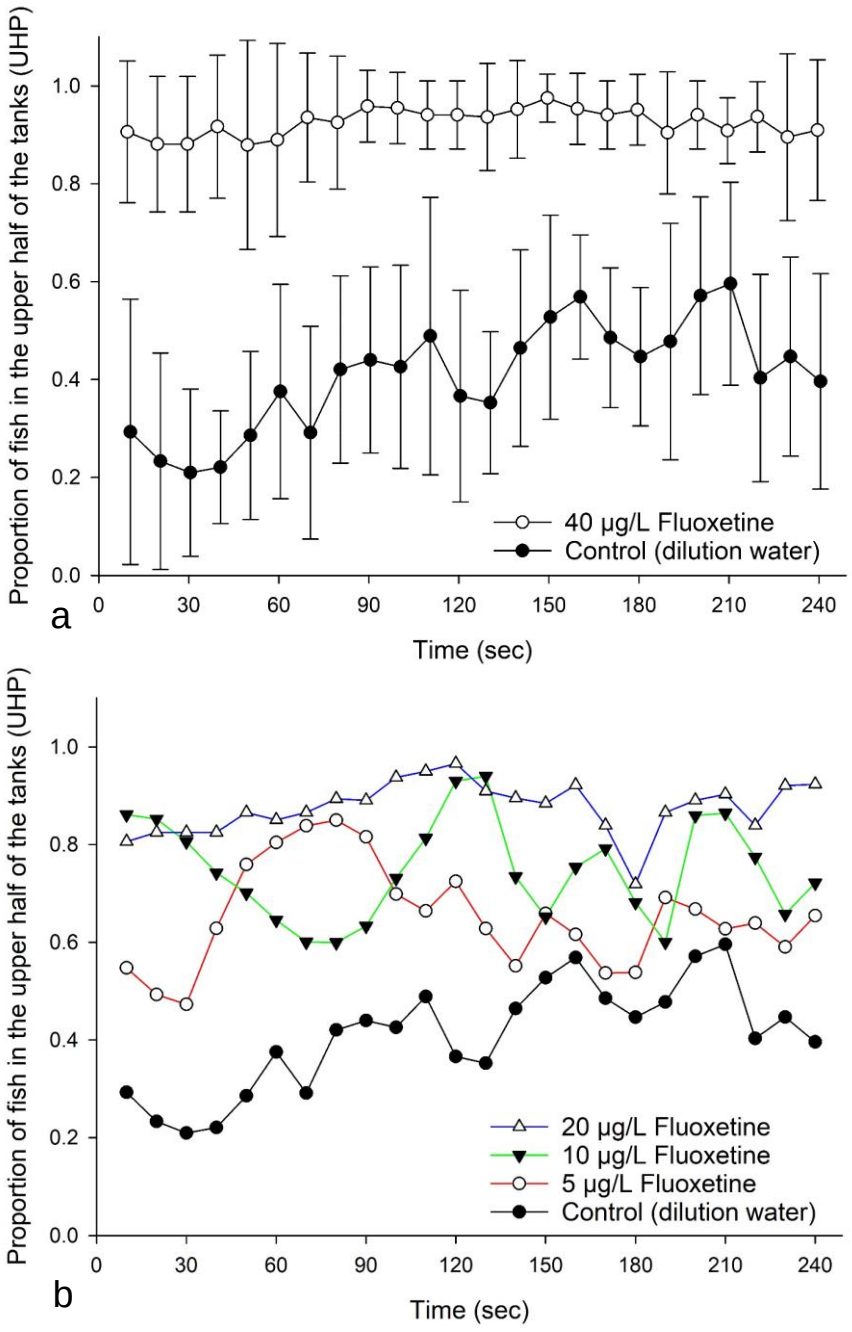

Fig. 7: Time course of the average upper half preference by control zebrafish (*Danio rerio*) and zebrafish exposed to fluoxetine over the first 4 min of all novel-tank tests conducted. (a) Exposure to 40 µg/L fluoxetine. Data are given as means ± SD of 10 observations in 4 replicate experiments (n = 4 × 10). The proportion of fluoxetine-exposed zebrafish in the upper half of the tank was consistently higher than in controls ($p < 0.001$; one-way ANOVA in combination with Tukey´s test for pairwise comparisons). (b) Exposure to 5 - 20 µg/L fluoxetine. Data are given as means of 10 observations in 4 replicate experiments (n = 4 × 10). In the majority of cases, the proportion of fluoxetine-exposed zebrafish in the upper half of the tank was consistently higher than in controls ($p < 0.001$; one-way ANOVA in combination with Tukey's test for pairwise comparisons). For the sake of clarity, data were only plotted without standard deviations.

Since the "novelty" aspect was most pronounced at the beginning of the test (i.e., immediately after release from the transfer net) and that habituation in control fish gradually developed from 30 sec, the analysis was focused on the first 2 min. Although variability in behavior was considerable, a full analysis of UHP over the initial 2 min after transfer confirmed the naked-eye observations and revealed a clear concentration-dependent increase in UHP (Fig. 8). After the initial period of 2 min, only the most prominent effects caused by 40 µg/L fluoxetine remained clearly distinguishable from the more and more explorative "normal" behavior shown by the controls.

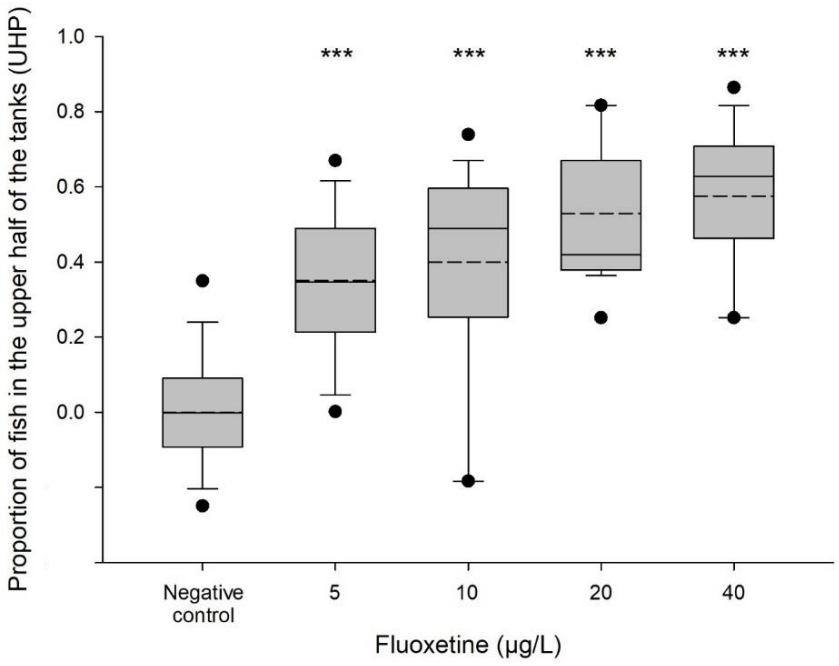

Fig. 8: Upper half preference of zebrafish (*Danio rerio*) after exposure to 5 - 40 µg/L fluoxetine in the novel tank test during the initial 2 min after transfer, revealing a clear-cut positive concentration-response relationship from the lowest test concentration of 5 µg/L fluoxetine. Data are given as boxplots for n = 120 observations × 4 replicates with the 25^{th} to 75^{th} percentiles (dashed and solid lines for average and median values, respectively; whiskers for 10^{th} to 90^{th} percentiles). Differences from negative controls by one-way ANOVA-on-ranks in combination with Dunn's *post-hoc* test: *** $p < 0.001$.

As an alternative presentation, the portion of UHP measurements (1 per second) above the negative control average can be computed for any given minute (Fig. 9): As might be expected, the negative control is evenly distributed and lies above the average in 42.0 ± 15.8 % of all samples. Depending on concentrations, fluoxetine exposure caused an increasing number of above-control measurements than might be expected by chance. Given that the number of data points considered is closer to the number of biological replicates (n = 4) than in Fig. 7, this alternative representation loses significance with regard to variability of the data and the statistical power and can thus not replace, but only complement Fig. 8.

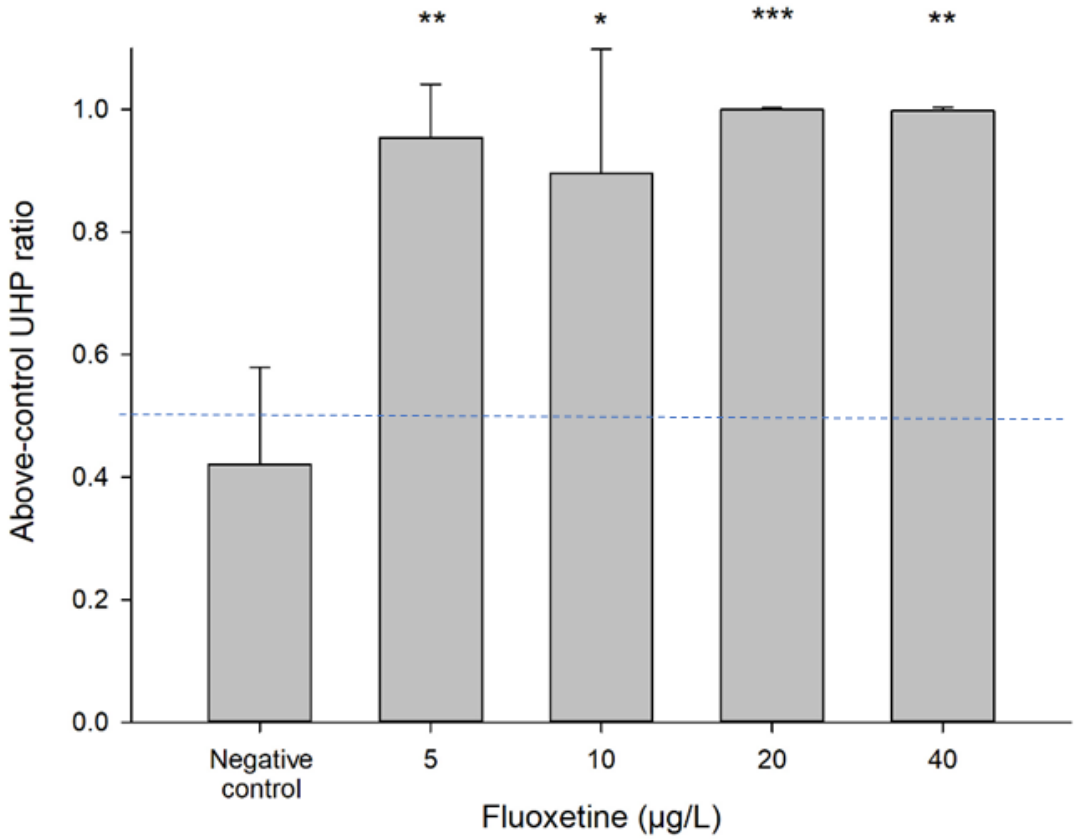

Fig. 9: Above-control portion of upper half preference (UHP) measurements as an alternative presentation of UHP of zebrafish (*Danio rerio*) after exposure to 5 - 40 µg/L fluoxetine in the novel-tank test during the initial 2 min. Data are given as columns representing the mean of 4 independent replicates measured at 1 and 2 min after release of the fish from the net (n = 4 × 2). The dotted line indicates a hypothetical "normal" negative control behavior (above-control mean 50 % of the time). Differences from negative controls by one-way ANOVA-on-ranks in combination with Dunn's *post-hoc* test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.3.2 Predator distance and shoal coherence were altered in a dose-dependent fashion

When confronted with the predator dummy, zebrafish from all treatment groups tended to move further away from the dummy than would be expected in a random distribution (score > 2.5; Fig. 10). Fluoxetine treatment apparently increased the average distance in a concentration-dependent fashion. Due to high variability, however, only effects at the highest concentration of 40 µg/L fluoxetine were statistically significant. In contrast to findings in the novel tank tests, these observations would (superficially) suggest increased anxiety in fluoxetine-treated zebrafish.

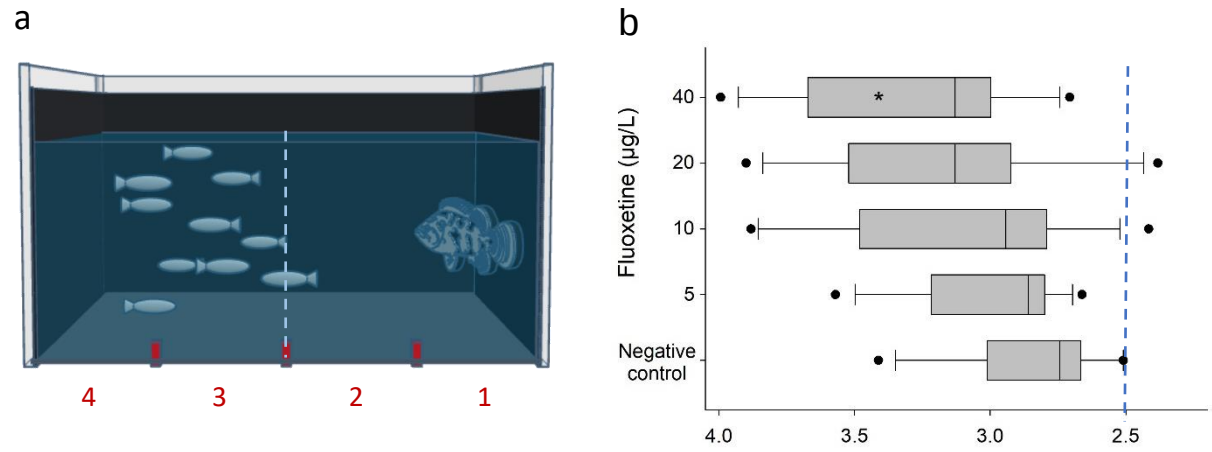

Fig. 10: Distance between zebrafish (*Danio rerio*) and a dummy predator in the right half of the tank over an initial phase of 3 min of confrontation. (a) Four vertical zones indicated by values from 1 (closest to the predator) to 4 (furthest away) were digitally applied to the video footage to quantify the behavior observed. (b) As a measure of avoidance behavior, data are given as horizontal boxplots of 3 time points (1, 2, 3 min) of 4 replicates (n = 3 × 4) with the 25th to 75th percentiles (solid lines for median values; whiskers for 10th to 90th percentiles). The dotted line represents the expected score for random swimming locations (evenly distributed around the center of the tank). Differences from negative controls by one-way ANOVA-on-ranks in combination with Dunn's *post-hoc* test: * $p < 0.05$.

Besides increasing the distance to the threat, the survival chances of prey fish is usually thought to increase by decreasing the distance to conspecifics ("hiding within the shoal"). Shoal coherence can be calculated as the number of vertical zones occupied by the shoal divided by the number of individuals forming the shoal (Fig. 11; also see Table 1), and lower scores would be expected to represent more cautionary behavior. Interestingly, mean coherence scores showed a trend opposite to the distance scores: There was a concentration-dependent trend towards increasingly loose shoals with increasing fluoxetine concentrations ("confusion"; Fig. 11a), which is, however, in line with the fluoxetine-typical effect of decreased anxiety (Zindler et al., 2019b, 2020b). A more-in-depth comparison of negative controls and zebrafish exposed to 40 µg/L fluoxetine with higher temporal resolution reveals a rapid differentiation of shoal coherence as early as 30 sec of predator presentation (Fig. 11b), indicating the opposite to what might be expected as a normal reaction of shoaling fish: The fluoxetine-treated shoals did not only fail to cohere more tightly after introduction of the predator dummy, the individuals rather appeared to actively spread out the shoal. A very similar pattern was observed with fish exposed to 10 or 20 µg/L fluoxetine (Fig. 11c).

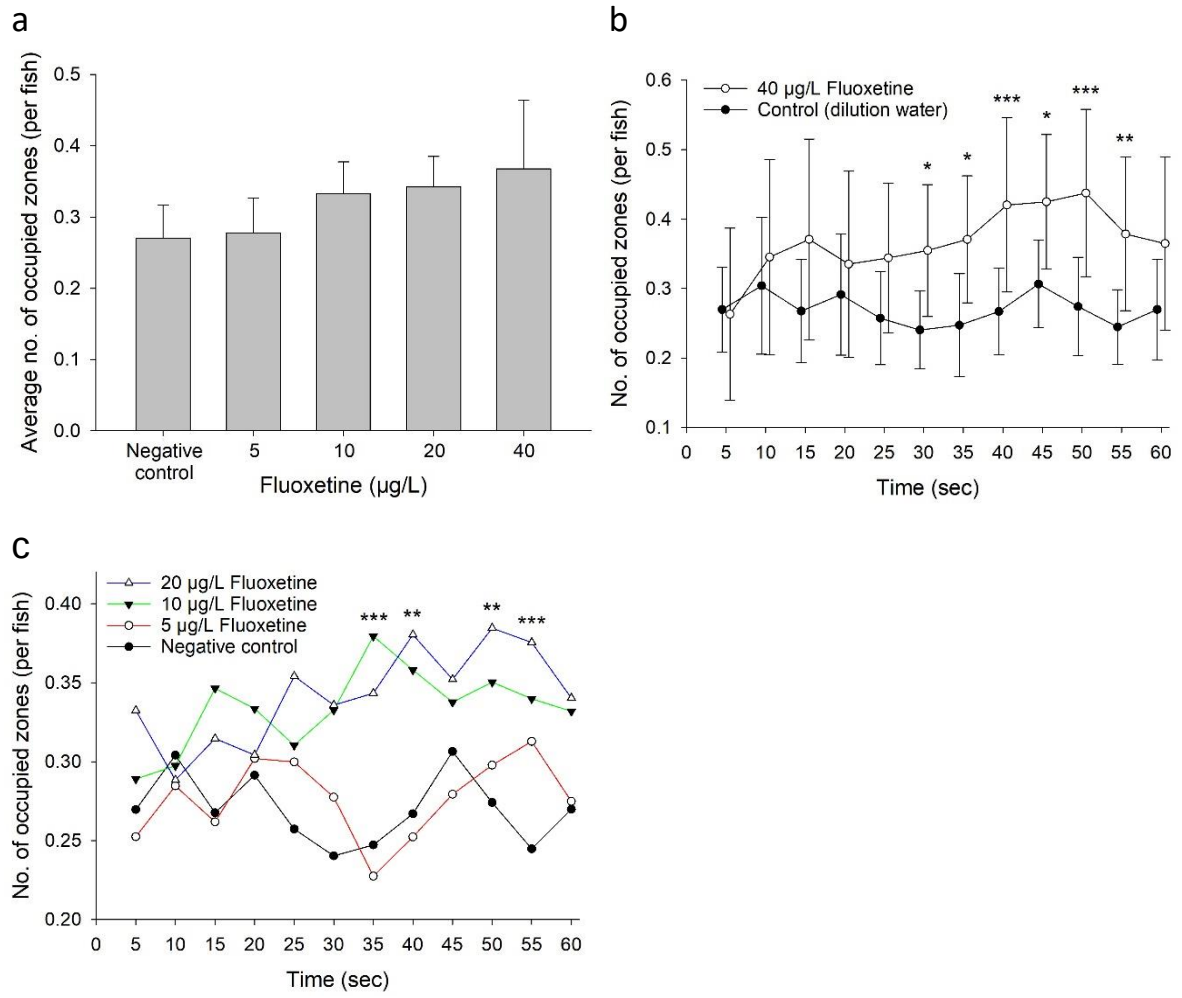

Fig. 11: Effect of exposure to 5 - 40 µg/L fluoxetine on shoal coherence behavior of zebrafish (*Danio rerio*) within the first minute of confrontation with a predator dummy. Since a lower score (fewer zones occupied per fish) relates to a tighter shoal and *vice versa*, fluoxetine exposure induces a decline in anxiety behavior in zebrafish (cf. Zindler et al., 2019b, 2020b). (a) Shoal coherence scores of zebrafish relative to fluoxetine concentrations (n = 4). (b) Higher temporal resolution (five-second means; n = 4 × 5) of shoal coherence of negative control zebrafish *versus* zebrafish exposed to 40 µg/L fluoxetine reveals that differences are established from ≥ 30 sec. (c) Shoal coherence scores of zebrafish exposed to lower fluoxetine concentrations (0 - 20 µg/L). Error bars were omitted for the sake of clarity. Significance of differences from negative controls by one-way ANOVA in combination with Tukey's test for pairwise comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.4 Discussion

The exposure scenario used for the two behavior tests proved more sensitive than previous approaches: The effect concentration of 40 µg/L is among the lowest levels of fluoxetine to produce statistically significant effects on zebrafish, which are usually in the range above 100 µg/L (Cachat, 2013, Egan et al., 2009, Wong et al., 2010). To the best of my knowledge, behavior alterations after exposure to ≤ 5 µg/L, which was still effective in my novel tank tests,

have so far only been described in other species such as Siamese fighting fish (*Betta splendens*), Arabian killifish (*Aphinius dispar*) or Japanese medaka (Oryzias latipes; Ansai et al., 2016, Barry, 2013, Dzieweczynski and Hebert, 2012).

5.4.1 Reflections on apparatus design and data acquisition

The novel tank test in particular proved to be well suited for my shoal behavior approach, revealing a prominent anxiolytic-like top preference, as would be expected after fluoxetine treatment (Stewart et al., 2014a). The 8 L tank, which is more than 5 times as large as common models (Cachat et al., 2011), has apparently provided sufficient space for the increased number of test subjects. Both ways of quantification – upper half preference (UHP) and above control UHP ratio – proved similarly effective for the current data set, but might of course reveal particular strengths or weaknesses when applied to differently distributed results.

Although I have not run additional trials with even simpler predator dummies like a centrifuge tube (Gerlai et al., 2000), results indicate that the plastic leaffish was indeed perceived as a threat and produced measurable effects on the shoal distance, which is intuitive and relatively easy to assess with the zone approach, but also surprisingly difficult to affect (Bass and Gerlai, 2008).

The attempt to also calculate an approximation of shoal coherence (or cohesion) from the same zone data set is, of course, associated with an important simplification: Whereas most studies sacrifice depth of information for the sake of practicality and measure neighbor distances in a "flattened" top view rather than three-dimensionally (Buske and Gerlai, 2011, Miller and Gerlai, 2008), I went one step further and only approximated the extent of the shoal in a single dimension (horizontally). Although this approach did allow me to detect an effect at the highest utilized fluoxetine concentration, comprehensive 3D information might also reveal less prominent effects, if only calculation were feasible. Without this option, using a limited number of zones rather than measuring "exact" distances between fish in the camera feed is not only less labor-intensive, but also greatly reduces the impact of errors from constellations that only seem close in 2D.

With respect to the workload, the frame-by-frame review of recordings, which is necessary to correctly count the fish in each zone, is indeed tedious. However, the data that can be gathered this way are certainly easier to quantify than the classic manual recording of events, and subjective differences between observers such as the lag between occurrence and recording can be avoided. A strict separation of the observer from the fish during trials also helps to allow more natural behavior (Egan et al., 2009). The detailed analysis of only one frame per second proved sufficient; a higher temporal resolution would unnecessarily increase the workload. However, more frames should always be recorded (at least 5 fps) to provide a reservoir of frames for a better distinction of fish in the focal frame (Miller and Gerlai, 2007).

Although I was able to show that several "classic" endpoints from the respective tests could easily be adapted from isolated adult individuals to shoals of larval to juvenile zebrafish, I also experienced certain difficulties: A common aspect of the behaviors selected is their simplicity due to the zoning approach – with single fish and markings on the tank, they might even be

recorded manually in real time without need for precise measurements. However, not all zone-related observations were found to yield meaningful results when applied to a group of test subjects. The common novel tank endpoint "latency to top", for example, proved to be problematic, since it was often triggered by single individuals who explored the top briefly and seemingly by chance. Similar problems occurred when counting the number of "transitions to top", a parameter often found in single-fish novel tank studies. In particular, I found that individual fish exposed to high fluoxetine concentrations often remained near the surface instead of reliably diving at the beginning of the recording. Thus, an evaluation of transitions to the top half was misleading (after delayed diving and subsequent return) or in some cases not possible at all.

Measuring additional behavioral traits such as freezing might reveal even more about the test substance's likely effects in a critical situation, but these movement-related patterns are much more difficult to see in a series of still images than the location-dependent metrics used in this study; in fact, recording such additional parameters would drastically increase the workload. Durations of top visits (Stewart et al., 2013) as well as general activity could best be measured if individuals were tracked during the entire test, which proved almost impossible without automated 3D analysis. However, it has also been argued that freezing and erratic movements may largely originate from experimental setup and handling rather than substance exposure (Rosemberg et al., 2011).

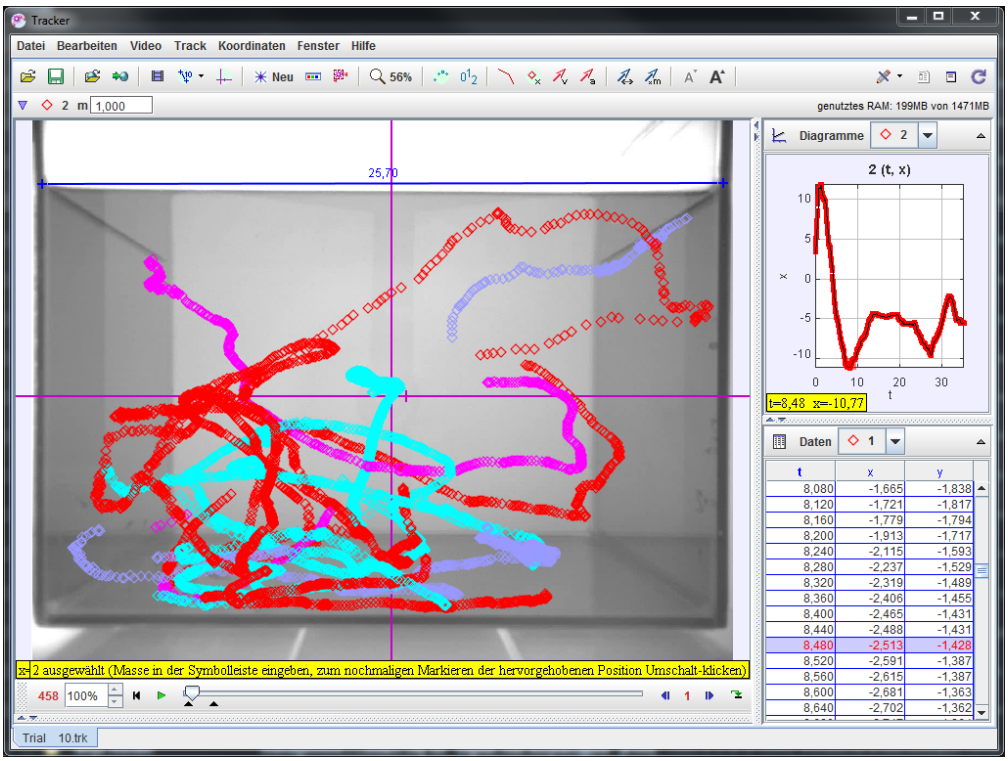

Fig. 12: One of several futile attempts to track the juvenile fish with special software (in this case, "Tracker"). This application is capable of rudimentary motion tracking and accepts user input in more complex situations, if recognized as such (note the track gaps near the center where the program likely confused different fish). Unfortunately, the amount of necessary manual corrections increased drastically whenever fish swam less than one body length apart and could not seem to handle more than four fish per 5-minute video.

To the best of my knowledge, tracking of free-swimming groups of more than five juvenile or even larval zebrafish has so far not been documented in literature: Even if the problems arising from the small size of the fish are resolved, individuals frequently intersect for several video frames. After any such incident, the correct identities of both animals might be lost, falsifying multiple parameters and perhaps even misreading the apparent change as a stress-induced irrational movement. Under such dynamic conditions, automated tracking is prone to producing misleading data (Cachat et al., 2010; cf. Fig. 12).

On the other hand, entirely different endpoints classically applied to isolated fish could be adapted to my approach, e.g. the light-dark preference test (Maximino et al., 2007). I have not investigated this further, since this would require an altogether different arena, but it would certainly be interesting to see how shoals of fish perform under conditions of varying light intensity and which drug effects differ between the seemingly similar light-dark and the novel tank test (Kysil et al., 2017).

5.4.2 Apparent discrepancy between the two predator response endpoints

The anxiogenic- and anxiolytic-like results from the predator response tests (increased distance *versus* decreased shoal coherence) seemingly contradict each other and the anxiolytic-like top preference in the novel tank tests, but are actually in line with previous observations on the "serotonin syndrome": Especially serotonergic substances like fluoxetine may cause complex behavior profiles involving surfacing behavior in the novel tank (anxiolytic) as well as anxiogenic responses in other settings, resulting from a drug-induced state of general confusion and agitation (Stewart et al., 2013). Perhaps two completely different behaviors are currently described with the construct of "anxiety" without fully understanding the actual mental states of the animals (Maximino et al., 2012). Analysis of cortisol levels might have made the distinction clearer, but unfortunately the fish used were too small at the time of the behavioral assays (Cachat et al., 2010).

Another possible explanation for the apparent discrepancy would be a disruption of optical sensation, leaving the fish ignorant to much of their environment (novel tank) or the identity of their conspecifics (shoal coherence), while becoming all the more anxious about the movement of the predator dummy which they could still sense *via* the lateral line organ. However, no such effect of fluoxetine has been described – to the contrary, fluoxetine has even been reported to be capable of healing certain visual impairments (Sharif et al., 2019).

5.4.3 Applicability of the basic concept – an interim conclusion

Endpoints related to behavior can easily be implemented into the current protocol for the fish early-life stage test according to OECD TG 210. Thus, additional population-relevant information can be collected without increasing the number of experimental animals. Of course, extra time is needed for behavior analysis in the aftermath of such an amended experiment, but the time expense in the laboratory hardly goes beyond the usual effort required for established flow-through setups. Yet, additional experiments with other classes of (neuro)toxicants are required to further optimize and validate the protocol for juvenile zebrafish behavior recording.

Further automation of the analysis may allow the implementation of additional metrics such as freezing behavior, but the small size and free movement of the test subjects pose technical difficulties. With the implementation of additional behavioral parameters, the risk of overlooking important aspects of behavior modification by chemical agents could be minimized. Regarding political requirements to reduce the number of experimental animals and to reduce the suffering of animals during experimentation according to the 3R principles (at least in Europe and the U.S.), further efforts are required to include especially younger developmental stages into behavioral studies. However, if behavioral changes as highly important population-relevant endpoints were to be implemented into existing guidelines, this will probably not be possible with true alternative (animal-free) testing methods in the foreseeable future.

6. Application of the new test procedure to treatments with carbamazepine, paraoxon-methyl and tris(1,3-dichlorisopropyl)-phosphate (TDCPP)

6.1 Introduction

Chapter 5 illustrated the development of a novel tank test and a predator avoidance test with juvenile zebrafish (*Danio rerio*) and the model neurotoxicant fluoxetine. Since fluoxetine, a selective serotine reuptake inhibitor (SSRI), could be shown to have strong effects in either approach, this substance also served as a positive control in the subsequent study, which was designed to demonstrate the applicability of the two novel test protocols to neurotoxicants with other modes of action. Out of the reference compound list of NeuroBox, carbamazepine, paraoxon-methyl, and tris(1,3-dichloroisopropyl) phosphate were selected as examples of pharmaceuticals, organophosphate insecticide metabolites and flame retardants (endocrine disruptors) with potential effects on neural development, respectively. In order to minimize interference with unspecific secondary effects, EC_{10} concentrations from range-finding experiments based on the fish embryo test (FET) according to OECD TG 236 (OECD 2013) were used as maximum test concentrations (cf. Kais et al., 2015).

Carbamazepine (CBZ) is a dibenzazepine-type anticonvulsant drug used primarily in the treatment of epilepsy and neuropathic pain, which is frequently found in the effluents of wastewater treatment plants due to low microbial degradation or photolysis rates and only limited attachment to sludge (Ferrari et al., 2003, Zhang et al., 2008). Nearly a third of the administered drug dose leaves a patient unchanged, finding its way into the water cycle as feces (Zhang et al., 2008). As one of the four major antiepileptic drugs in current use, carbamazepine can frequently be found in surface water at near µg/L concentrations and has been detected in U.S. drinking water at concentrations up to 10 ng/L (Ambrósio et al., 2002, Benotti et al., 2009, Metcalfe et al., 2003, Pfluger and Dietrich, 2001). Carbamazepine preferentially binds to and blocks voltage-gated sodium channels (Ambrósio et al., 2002, Macdonald, 1989) and also affects the brain arachidonic acid cascade, leading to its secondary indication as a mood stabilizer (Rao et al., 2008). There are a number of other less understood receptor or channel interactions, e.g. with voltage-gated Ca^{2+} and K^{+} channels, which may also contribute to the drug's mechanism of action especially at higher concentrations (Ambrósio et al., 2002). Like most antiepileptic drugs, CBZ has been linked to developmental neurotoxicity in humans and animal models (Beker van Woudenberg et al., 2014). Since the 96 h EC_{10} for zebrafish has been reported as 167 µM (39.4 mg/L; Beker van Woudenberg et al., 2014) and Ferrari et al. (2003) found a 10-day early life-stage LOEC of 50 mg/L, 40 mg/L were used as the highest test concentration.

The organophosphate insecticide metabolite paraoxon-methyl (PXM) was chosen for its well-known effect as an irreversible acetylcholinesterase (AChE) inhibitor (Kais et al., 2015, Teixido et al., 2013). PXM is the active metabolite of the now widely prohibited organophosphate insecticide parathion-methyl and about ten times more toxic than its parent compound (De Schryver et al., 1987, Dzyadevych et al., 2002). Through phosphorylation of the active site of

AChE, PXM renders the enzyme unable to degrade the neurotransmitter acetylcholine at postsynaptic sites, resulting in convulsions and hypertonic paralysis (Kais et al., 2015, Küster and Altenburger, 2006, Sánchez-Santed et al., 2004, Walker, 2003). Ultimately, vertebrates exposed to sufficiently high doses often die of respiratory failure, but small doses can already have long-lasting effects due to the slow reactivation (or rather, re-synthetization) of AChE after the toxin itself has long been neutralized (Walker, 2003). The exposure route to fish would usually be run-off or spill events from treated farmland (Küster and Altenburger, 2006), and workers with contact to the pesticide have been found to increasingly develop cognitive issues as well as Alzheimer's or Parkinson's diseases (Baldi et al., 2003, Grandjean and Landrigan, 2006). The 96 h EC_{10} for *Danio rerio* has been reported as 2 mg/L (Kais et al., 2015).

As an example of an endocrine disruptor with potential effects on neural development, tris(1,3-dichloroisopropyl) phosphate (TDCPP; also known as TDCiPP), is an organophosphate flame retardant widely used in materials such as polyurethane foams (Dasgupta et al., 2018, Dishaw et al., 2014b, McGee et al., 2012). Among other effects, TDCPP has been linked to changes in the hypothalamic-pituitary-thyroid (HPT) axis, which affects neurodevelopment (Dishaw et al., 2014b). While early exposure appears to be most potent, possibly through alterations of zygotic remethylation, the consequences may last a lifetime (McGee et al., 2012, Oliveri et al., 2015). Organophosphate flame retardants, especially TDCPP, are highly persistent in the environment and are barely degraded in sewage treatment plants (Dishaw et al., 2014a, van der Veen and de Boer, 2012). It is, therefore, not surprising that Benotti et al. (2009) found much higher concentrations of TDCPP in U.S. drinking water samples (median 220 ng/L) than they did of CBZ. Considerable accumulation has been identified in sediments (van der Veen and de Boer, 2012) posing a potential risk to fish eggs and other bottom-dwelling organisms. Based on the data by McGee et al. (2012), the 96 h EC_{10} for TDCPP lies around 2 µM (0.86 mg/L).

In the following, these three substances with distinct modes of action shall be used to verify the effects described in chapter 5.

6.2 Materials and methods

6.2.1 Egg collection and rearing

As described in chapter 5, eggs for experimental use were obtained from genetically heterogenic spawning groups as described in OECD TG 236 (OECD, 2013b) and allowed to hatch in 1.7 L polycarbonate tanks (Tecniplast, Italy) within a 26 °C incubator (Binder, Tuttlingen, Germany). Large batches were split among several such tanks so that each held less than 300 eggs, and water was exchanged daily to ensure good quality. From the 5th day post-fertilization (dpf), larvae were fed with *Paramecium caudatum* (own culture) twice daily *ad libitum*. At 6 dpf, larvae were transferred to 21.5 L full-glass rearing aquaria (18 × 40 × 30 cm) supplied with a continuous 1× flow-through water replacement and gradually received larger food particles: from day 9 to 11, larvae were fed with *Paramecium*, Nobil fluid 'Artemia' (JBL, Neuhofen, Germany) and Micron powder (Sera, Heinsberg, Germany). From day 12 to 15, they were given *Artemia* nauplii in addition to *Paramecium*, Nobil fluid and Micron powder, before *Paramecium* was removed from their diet from day 16. All steps were conducted under the same water quality and light regimes as described at the outset.

Zebrafish from distinct breeding groups were raised in fresh water until 21 dpf, at which point they were randomly divided into groups of 10 individuals and transferred to glass exposure tanks.

6.2.2 Substance exposure

All test substances were dissolved in deionized water except carbamazepine, which was dissolved in deionized water with 0.6 % DMSO as solvent. The dilution and delivery of compounds to the 5 L exposure tanks was achieved by gravity *via* rotameters (Yokogawa, Ratingen, Germany) for fresh water and peristaltic pumps (Gilson, Limburg an der Lahn, Germany) for the stock solutions which were prepared freshly every four days. A permanent 21-fold dilution of the stock solutions led to a constant flow-through of the specified nominal concentrations.

Each experiment comprised a negative control group, a group treated with 40 µg/L FLX (positive control), and four groups treated with increasing concentrations of one of the other model neurotoxins: 1.25, 2.5, 5 and 10 mg/L for CBZ, 0.0625, 0.125, 0.25 and 0.5 mg/L for PXM and 0.12, 0.24, 0.48 and 0.96 mg/L for TDCPP. All exposures were carried out in duplicate with two full volume exchanges (10 L) per day. In order to reduce the workload on days of behavior recording, the two replicates were set one to two days apart. Flow-through rates were checked at least once a day.

6.2.3 Behavior testing and analysis

Details of the observation tank (including the involved cameras and software), test procedures, euthanization, video and data analysis were essentially the same as described before (see materials & methods of chapter 5).

The non-availability of a suitable automated solution for tracking the juvenile fish remained a problem so I once again manually recorded the approximate fish positions by applying standardized graphic templates for two (top/bottom; novel tank) or four (vertical; predator response) zones to every tenth frame of the recording (one per second). A portion of this video "transcription" was delegated to research assistants in order to save time and to evaluate whether different observers would arrive at the same results.

6.3 Results

6.3.1 Increased upper half preference in novel tank tests

The positive control fluoxetine (FLX) as well as carbamazepine (CBZ) and paraoxon-methyl (PXM) clearly caused an increase in upper half preference (UHP) over negative controls. With a mean increase of 0.67, the effect of FLX was exceptionally clear ($p < 0.001$), which justified the role of FLX as a positive control substance (Fig. 13a). CBZ also caused a notable increase of ~ 0.2 during the second half of the initial minute of novelty stress, with a maximum difference of 0.31 (CBZ mean 0.19 *vs*. control mean of -0.12) about 50 seconds into my experiments (Fig. 13b). Likewise, the UHP of the PXM treatment groups was increased towards the end of the

first minute (Fig. 13c), averaging about 0.1 above negative controls with a maximum mean difference of 0.15 around 55 seconds ($p < 0.001$).

In contrast, TDCPP did not induce statistically significant differences from controls. There was only a trend towards slightly increased upper half preferences, which, however, remained within the standard deviation of the negative controls.

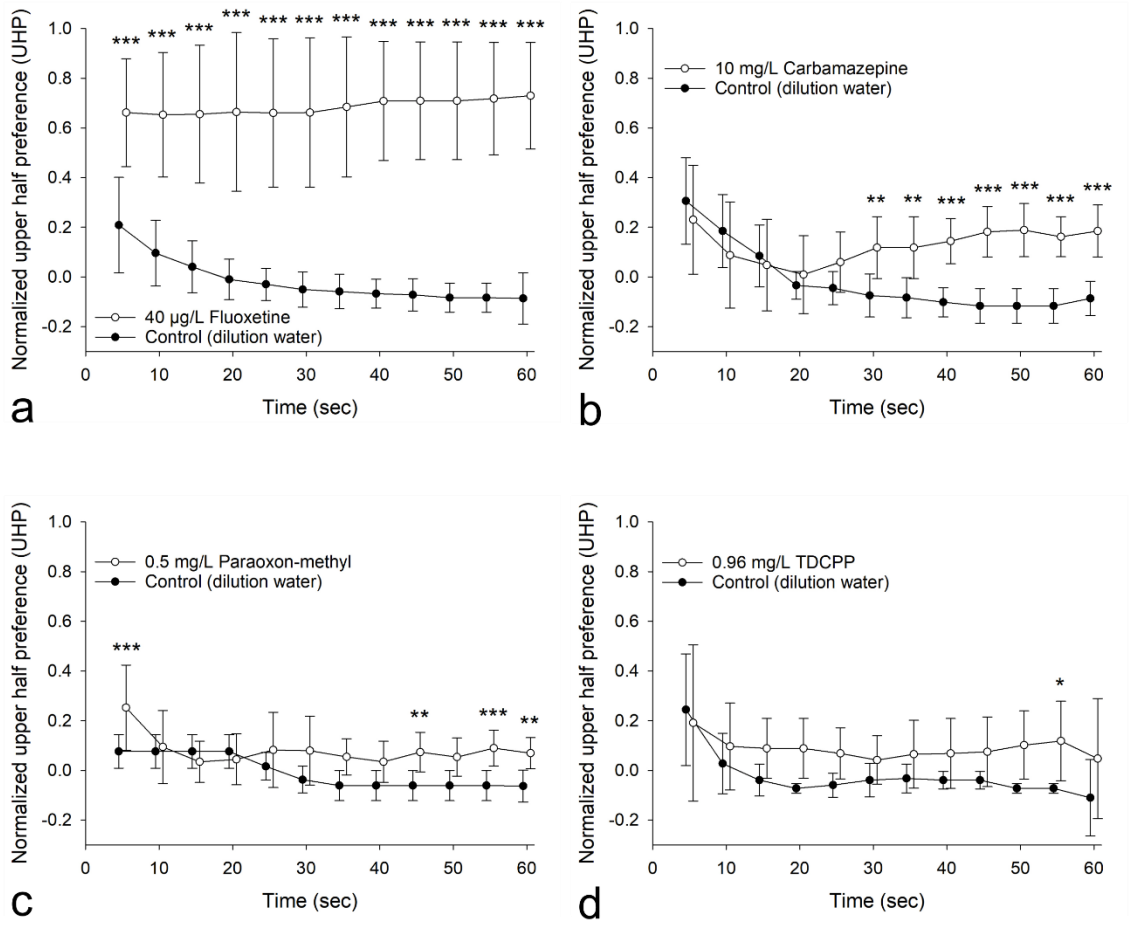

Fig. 13: Time-course of the upper half preference of 35 d old zebrafish (*Danio rerio*) larvae in the novel tank test after treatment with fluoxetine (a; positive control), carbamazepine (b), paraoxon-methyl (c) and TDCPP (d) normalized to the negative controls. Data are shown for the highest tested concentration of each substance. Measurements were made every second and pooled into 5-second averages over the initial minute of the tests. The scores were normalized to the control average of each subset during this period. Depending on the number of independent replicate experiments, each dot represents a total of $n = 10 \times 5$ (FLX), $n = 4 \times 5$ (TDCPP) or $n = 3 \times 5$ (CBZ, PXM) measurements. Error bars indicate standard deviations. Differences from negative controls by one-way ANOVA-on-ranks in combination with Tukey's test for pairwise comparisons: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Typically, the variance of data increased during the second minute of recording, but apparently independent of the concentration (details for concentrations not shown). To account for this observation and the increase in the number of data points to plot, Fig. 14 provides an alternative presentation. This illustrates the increase of the upper half preference induced by exposure to the various test compounds more clearly, although at the cost of a loss of information about the time-course.

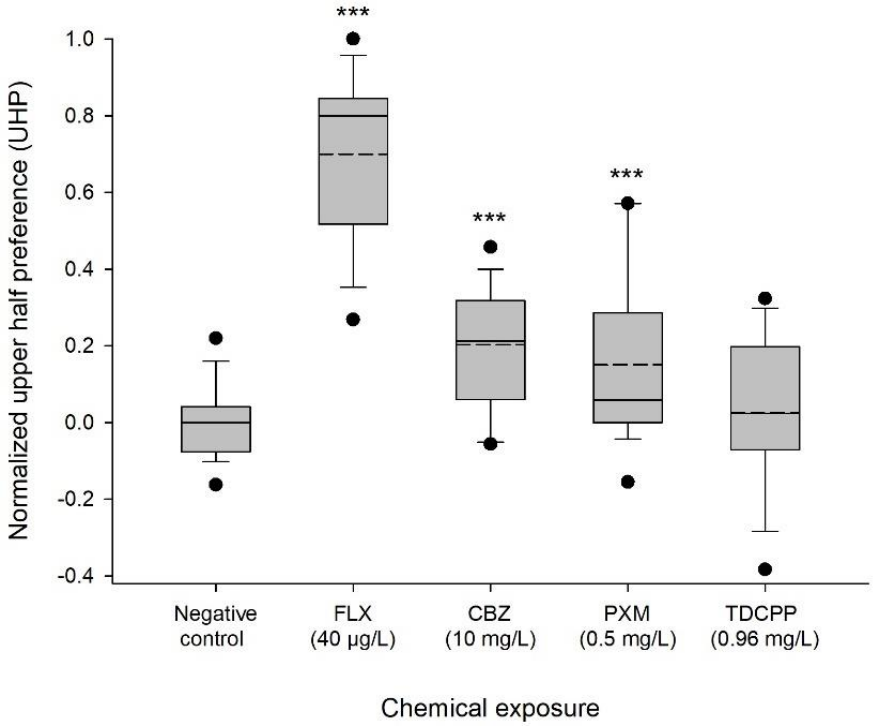

Fig. 14: Distribution of upper half preference data cumulated over the first two minutes of the novel tank test recordings relative to controls. Shoal locations were assessed once per second (n = 120 per replicate) and normalized to the per-minute average "background" behavior of the respective negative control group from the same batch of eggs. Boxes indicate the 25th to 75th percentiles of measurements, with the dashed and solid lines identifying the average and median values, respectively. Whiskers encompass the 10th to 90th percentiles. Number of independent replicates: n = 9 for negative controls & FLX; n = 3 for CBZ, PXM and TDCPP. Differences from negative controls by one-way ANOVA-on-ranks in combination with Dunn's *post-hoc* test: *** $p < 0.001$.

Visualizing every per-second measurement from the first two minutes (i.e., 120 per replicate experiment), the box-plot identifies clear differences between treatments that exceed the level of variance. As the data were normalized to account for "normal" background behavior, mean and median for the dilution water controls are approximately 0. In agreement with Fig. 13, the UHP is clearly increased after exposure to FLX, CBZ or PXM ($p < 0.001$), but TDCPP fails to cause a significant effect compared to the negative control.

6.3.2 Reductions in predator distance or shoal coherence

The predator response assays that immediately followed the novel tank tests were scored by means of four vertical zones instead of a top and bottom half (cf. chapter 5). I did not check for preferences for any single zone but assigned numerical values from 1 (closest) to 4 (furthest from the predator dummy) to each observed fish, resulting in average "distance scores" also ranging from 1 to 4. There was an overall tendency to move away from the predator's zone (Fig. 15): A randomly distributed group would produce scores around 2.5 in this experiment (cf. blue dashed line), but the medians of the controls and most exposure groups were above 2.78.

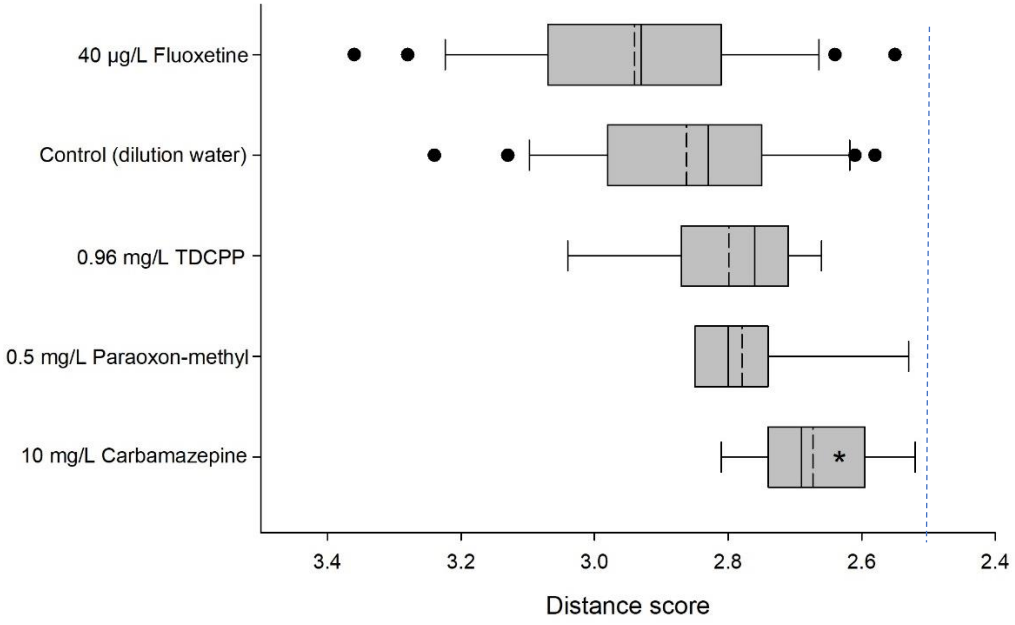

Fig. 15: Distance between test fish and a predator dummy over the initial three minutes of confrontation. From each replicate experiment, one average "distance score" per minute (n = 3 measurements per replicate; no. of total replicates: fluoxetine & control = 9 × 3; TDCPP, paraoxon-methyl & carbamazepine = 3 × 3) was considered as a measure of avoidance behavior. Scores are based on swimming locations in zones from 1 (closest to the predator) to 4 (furthest away). Boxes indicate the 25th to 75th percentiles of measurements, with the dashed and solid lines identifying the average and median values, respectively. Whiskers encompass the 10th to 90th percentiles. The dotted blue vertical line represents the expected score (2.5) for random swimming across all four zones. Differences from negative controls by one-way ANOVA-on-ranks in combination with Dunn's *post-hoc* test: ** $p < 0.01$.

The notable exception are the CBZ-affected fish which on average swam less far away from the predator. They did stay beyond the hypothetical threshold of 2.5, but came significantly closer to the simulated threat than those of the dilution water control cohort (median 2.69, mean 2.67, $p < 0.01$). FLX, which had served as a reliable positive control in the novel tank tests, did not appear to influence predator avoidance. There is a slight trend to move further away from the predator than the control groups, as also reported in chapter 5, but the difference between the two medians was not statistically significant (2.91 vs. 2.87).

All shoals kept in motion throughout the entire duration of the tests, and single fish did even enter the predator dummy's zone from time to time regardless of the treatment, indicating that increased distance alone is not what defines a healthy predator response. Therefore, shoal coherence was analyzed as an additional parameter. Based on the number of fish and the occupied number of vertical zones, I arrived at coherence scores that roughly described how loose (high score – shoal spread over many zones) or tight (low score) the shoal appeared at the given timepoint. As an example, a tight shoal of 10 fish may occupy one or two zones, leading to a score of 0.1 or 0.2 "zones per fish", respectively. The same shoal, distributed over all four zones, would yield a score of 0.4.

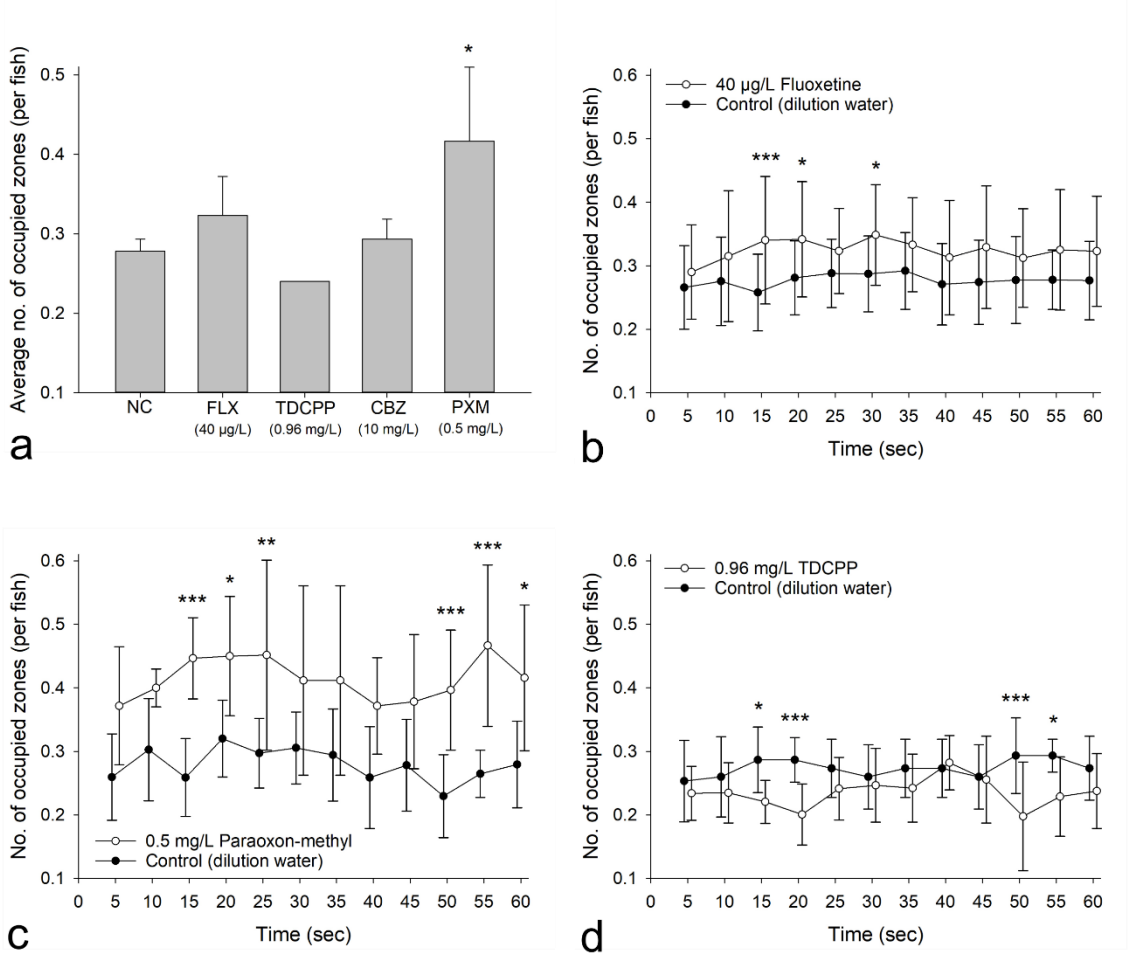

Fig. 16: Spread of the shoals of 35 d old zebrafish (Danio rerio) larvae upon confrontation with a predator dummy. A lower score indicates tighter shoaling (fewer zones are occupied per fish in the shoal) and vice versa. Panel a: Average shoal coherence during the first minute of all predator response tests, one mean per replicate (n = 9 for NC and FLX; n = 3 for TDCPP, CBZ and PXM). Panels b-d: Detailed time-course comparisons of substance treatments and their corresponding control runs for fluoxetine, carbamazepine and TDCPP. Data are given as 5 sec means from each replicate experiment made up the illustrated data points. Differences from negative controls by one-way ANOVA-on-ranks in combination with Tukey's test for pairwise comparisons: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The analysis of shoal coherence showed no statistically significant differences in average scores by the minute, although PXM exposed fish were scored higher (0.42 ± 0.09) than the other treatment groups and the dilution water control (0.28 ± 0.02; Fig. 16a) indicating that PXM shoals appeared to be less coherent. The coherence behavior after CBZ exposure was indistinguishable from the dilution water control (data not shown), but a closer look at the time course of the response of the other treatment groups revealed potentially interesting stretches.

As with the predator distance, FLX hardly altered the shoal coherence compared to the corresponding controls. There seemed to be a slight trend towards reduced coherence upon exposure, but only one 5-second mean exceeded the expected variation from the control (10 - 15 sec, $p < 0.01$; Fig. 16b). PXM, on the other hand, had a rather clear effect on this endpoint as was already suspected from the overall means in panel A: there were two 15-second periods of statistically significant reduction of the shoal coherence over the span of the first minute of all tests ($p \leq 0.05$; Fig. 16c). The overall trend of reduced coherence – i.e., higher scores – was also clearly visible in the time course. TDCPP was the only tested substance to show a trend of increased coherence compared to the associated controls (Fig. 16d). Although not enough to influence the overall result (cf. panel A), there were two 5-second intervals with statistically significantly tighter shoaling than in the dilution water controls ($p < 0.001$).

6.4 Discussion

6.4.1 Relative sensitivities of the juvenile behavior assays

A more-in-depth analysis of results from the novel tank test and the predator response assay with respect to statistical robustness and sensitivity reveals that both the lower detection level (LOECs) and the ability to statistically discriminate effects are comparable for both tests (Table 2).

Table 1: Relative sensitivity of endpoints in the behavior assays with juvenile zebrafish (*Danio rerio*) for the four model substances carbamazepine, fluoxetine, paraoxon-methyl and tris(1,3-dichlorisopropyl) phosphate (TDCPP) at 35 dpf. Data given as lowest observed effect concentrations (LOEC); deviations from negative controls: ↑(↑) = (significant) increase, ↓(↓) = (significant) decrease. For shoal coherence, an "increase" refers to a lower number of zones per fish. Grey fields indicate that no effective concentrations could be found (LOEC > highest tested concentration). Statistical significance: * $p < 0.05$; *** $p < 0.001$.

Method	Carbamazepine	Fluoxetine	Paraoxon-methyl	TDCPP
Novel tank test: upper half preference	10 mg/L ↑↑ ***	≤ 5 µg/L[†] ↑↑ ***	0.5 mg/L ↑↑ ***	0.96 mg/L ↑
Predator response test: distance	10 mg/L ↑↑ *	40 µg/L[†] ↑	> 0.5 mg/L	> 0.96 mg/L
Predator response test: shoal coherence	> 10 mg/L	40 µg/L ↓	0.5 mg/L ↓↓ *	0.96 mg/L ↑

[†] result from chapter 5

Fluoxetine was an exception as it induced particularly sensitive reactions in the novel tank test but no statistically significant effect on either endpoint of the predator response assay.

6.4.2 The novel tank test is sensitive to most model compounds

Despite considerable variability of results, the present study was able to demonstrate that the novel tank test with shoaling zebrafish can be applied to treatments other than the positive control fluoxetine (FLX); rather, the assay is capable of detecting more subtle effects than those by FLX (cf. Table 2). Using upper half preference as an endpoint in combination with various ways of analysis, different effects could be identified: besides the very potent FLX, carbamazepine (CBZ) had the most prominent effect on the time course of the novelty response (Fig. 13). Both CBZ and paraoxon-methyl (PXM) caused significant changes in the overall distribution of fish during the first minutes after transfer, regardless of the risk associated with top dwelling in an unfamiliar environment (Fig. 14). The relatively high peak value of 0.15 for PXM in the novel tank test was most likely caused by outliers (median only 0.06 above controls); therefore, although statistically significant, the "effect" caused by PXM exposure may not be ecologically relevant.

The lack of clear effects by TDCPP may be due to two factors: (1) Organophosphates have been shown to alter rather specific behaviors; the underlying mechanisms, however, are not yet known. As a consequence, wrong locomotion parameters might have been chosen, whereas other behavioral endpoints might well have shown changes by TDCPP (Oliveri et al., 2015). (2) The prime window of susceptibility to TDCPP might well be within earlier stages of zebrafish development so that the selected exposure frame was simply too late. In fact, McGee et al. (2012) found exposure at the onset of cleavage, i.e. within two hours instead of weeks, to be responsible for most developmental toxicity caused by TDCPP. In addition, my attempts to avoid interference with acute toxicity might have resulted in concentrations too low to cause effects by TDCPP in the given exposure scenario.

6.4.3 Predator response testing may be refined for more tangible data

The predator response assays produced less convincing results than the novel tank tests (cf. Table 2). In contrast to the initial results described in chapter 5, where FLX led to an increased predator distance, the analysis of distance scores revealed no significant difference between control and FLX-treated fish. CBZ exposure did lead to a significantly decreased distance, but the "normal" (control) response was not as clear as required to identify smaller deviations. Besides the variance of data mentioned above, which may well have concealed effects in the predator response test, a potential flaw in the setup could be identified: Maybe, the experimental tank needs to be longer to allow for a natural avoidance response. When confined to a small volume of water, zebrafish have been suggested to instinctively recognize the impossibility to stay outside the predator's striking distance and, therefore, to resort to other strategies (Ahmed et al., 2011). Unfortunately, such strategies would include erratic movements or freezing bouts, which are almost impossible to evaluate with my location-based approach and would require automated analysis (more on this later).

The constant desire of the fish to adjust their positions and to react to the uncomfortable proximity of the threat may also contribute to the high variance in distance. For future experiments, I therefore propose to increase the tank length to at least 50 cm.

One alternative to predator avoidance that I was able to evaluate is the formation of tight shoals in the face of danger: In contrast to most treatments with other test compounds, PXM significantly altered the degree of shoal coherence (or cohesion). The measurement of coherence might be improved by (1) assigning more than four distinct locations (zones) a fish can be allocated in and assigned to, and (2) considering the number of fish within each zone. The main reason for the simplification in the present approach was the ability to evaluate distance and coherence with the same set of manually scored data, i.e. maximizing labor efficiency. In an ideal setting, permanent tracking the location of each individual fish should be combined with precise data on the distances to each neighbor in a three-dimensional space. Yet, the results of the present study clearly document that a simplified approach may work in some cases.

By relating the zone preference to the number of fish, I attempted to reduce the variance caused by slightly different group sizes without actual effects on shoal coherence. The correction also reflects the increased probability of a larger shoal stretching over several zones, no matter how close the association between neighboring fish. This is only possible for similarly and adequately sized groups, though; If the number of fish is equal to or even lower than the number of zones, the scores become meaningless. In the present study, the PXM scores did not stand out based on such an overcorrection, but the absolute number of zones occupied was also significantly higher than that of the controls (0.5 mg/L PXM: 3.04 ± 0.67; dilution water: 2.57 ± 0.57).

It should be noted that there are ways to further improve the protocol for the predator response tests: Using a laboratory strain rather than wild-caught fish is the obvious choice from an animal welfare perspective, but it inherently bears the risk of using less wary test subjects. Although certain shapes and colors have been shown to trigger avoidance responses in fish that could never have met the modeled predator species before (Ahmed et al., 2012), it is possible that the extent of the response declines over generations of laboratory (in)breeding without any selection pressure on this behavior. Finally, the use of a simple predator dummy bears the risk that it might not be as effective as a more sophisticated dummy or even a live fish.

6.4.4 Variability in behavioral responses to chemical exposure

As a major caveat of laboratory-based behavior analyses, there is a permanent need to differentiate between natural behavioral patterns and artifacts resulting from rearing and breeding conditions. Genetics will certainly play an important role: different laboratory strains on the one hand and isolated populations in the field on the other hand are likely to show diverse reactions to the same challenge (Parichy, 2015). In the wild, populations show a wide range of aggression and boldness, e.g., when challenged with other zebrafish or predators. In captivity, however, aggression tends to increase (Martins and Bhat, 2014). This increase in aggressivity is not only an ancient response from wild fish that find themselves in an unfamiliar environment; it rather appears to be a trend in adaptation to typical laboratory conditions

(Oswald and Robison, 2008). In fact, laboratory-evoked behavior patterns, rather than increased or decreased fitness, have been shown to account for differences observed between strains (Egan et al., 2009). To account for genetic impact, (1) inbred strains might be used, or (2) only offspring from a single spawning group might be used for a given experiment in combination with an internal control from the same batch (this study). Although employing a strain with elevated baseline activity (e.g., increased anxiety in the zebrafish leopard strain) to better observe inhibitory effects might be beneficial especially in pharmacological research (Cachat et al., 2011, Maximino et al., 2013b), any selection of specific strains would have meant a diversion from my approach to mimic natural conditions.

Indeed, since the goal of the present study was the assessment of population-relevant behavior patterns, considerable variability of data is a price that needs to be paid for (more) realistic conditions. Variance might have been even higher if wild fish had been used. This, however, would have required even higher numbers of experimental fish to arrive at credible findings, resulting in conflicts with modern animal welfare considerations.

As another potentially disturbing factor, group size was considered: While fast-flowing streams may favor shoals of hundreds of individuals, an average of 11 zebrafish per shoal has been reported for slow or still water (Shelton et al., 2020) which was matched by my model groups. Higher stocking rates may increase aggression (Martins and Bhat, 2014) and stress, thus leading to another increase in variability (Cachat et al., 2010). Sex and age of experimental fish may also interfere with behavioral responses to drugs (Stewart et al., 2014b). Of course, any manipulation of the fish should be minimized, and the order of the experiments should be designed to avoid stress. In the present study, isolation of the fish prior to the novel tank test was avoided, and particular care was taken to transfer the fish to the novel tank as a group and as swiftly as possible. A third influence on the notoriously high variability in individual behavior responses (Cachat et al., 2010) might, of course, be the differential susceptibility of individuals to the pharmacological or toxic agents (Dzieweczynski and Hebert, 2012).

6.4.5 Difficulties associated with video analysis

Fine tuning of lighting conditions (e.g. direct sources as well as shielding from ambience) might help to further improve the initially described method (cf. Figs. 3 & 5). However, during the initial seconds of the novel tank trials, physical disturbances could not be excluded completely, thus preventing reliable automated analysis; sometimes such disturbances even caused problems during manual analysis for experienced human observers.

Although filtered water was used to refill the observation tank as recommended to avoid undesired particles (Blaser and Gerlai, 2006), the introduction of bubbles and debris could not be prevented completely when emptying the net during the introduction of the test fish. Whereas formation of minor bubbles cannot be avoided, one might consider to first transfer the fish to a sedimentation tank, where most of the debris is allowed to settle. This, however, would be in conflict with the strategy of reduce stress and handling time prior to the video recordings to a minimum.

Likewise, extraction of debris from the rearing tanks prior to transfer also represents stress and danger to the very small larvae and might also have an unpredictable impact on their behavior.

6.4.6 Suggested implementation of the new methods

As shown in the proof-of-concept with the selective serotonin reuptake inhibitor fluoxetine (FLX), behavior-related endpoints can easily be implemented into the current protocol for the fish early-life stage test (OECD TG 210) to collect additional important population-relevant information without increasing the number of experimental animals (see chapter 5). The present study clearly demonstrates the suitability of this approach to detect effects by neurotoxicants with different modes-of-action: FLX, the sodium channel inhibitor carbamazepine (CBZ) and the acetylcholine esterase inhibitor paraoxon-methyl (PXM). The fourth model compound, the known endocrine disruptor tris(1,3-dichlorisopropyl) phosphate (TDCPP), has largely failed to produce an effect most likely to the delayed onset of exposure from day 21; TDCPP may be expected to be most potent upon exposure from an earlier stage of development.

In addition to earlier findings, FLX had a strong and clear effect on upper half preference in the novel tank test but failed to reproduce its significant effect on predator distance. The trend towards an increase in distance after FLX exposure is not ideal for a potential positive control, since increased distance from the predator would be the reaction expected for the negative control group. Therefore, for future predator experiments, CBZ is recommended as a positive control. For the novel tank test, however, FLX remains a superb choice as a positive control.

Despite recent advances in technical development, the recording of toxicant-related changes in behavior remains a time-consuming effort, and the additional value of novel endpoints needs to be weighed off against the increased time and effort required. Although not described in detail, good experiences have been made with the training of assistant human scorers particularly for the non-subjective zone approach. In contrast, endpoints that would lead to an even higher workload and an increased inter-observer variability, such as the timing of erratic movements, were excluded from the analysis. Finally, two different behaviors, predator distance and shoal coherence, could easily be extracted from the same set of location data without reviewing the video footage another time, allowing for the analysis of two independent, but equally informative predator response strategies. The fact that this approach did not result in a stereotype response pattern for the four model compounds illustrates the importance to analyze a variety of endpoints before a substance can be categorized safe.

If the method described here were to become a routine component of OECD TG 210 studies, it is imperative to develop a convenient hard- and software solution that allows the safe, simultaneous tracking of multiple free-swimming small fish in order to scale up considerably.

More important than researcher workload, on the other hand, is the fact that the basic concept of OECD TG 210 requires large amounts of test substances and the exposure of a high number of larval fish. The use of zebrafish in early life-stage exposures bears a critical developmental stage at around 10 – 12 days with sometimes increased mortalities even in controls. The present protocol attempts to mitigate the suffering of experimental animals by delaying the onset of exposure to 21 days, when a preselection of viable individuals has been completed. There is a strong trend towards animal-free alternatives in toxicity testing; however, if behavior remains a major source of information on potential neurotoxic properties of a substance, replacement of animal testing may still have a long way to go.

7. All about sensitivity? Comparison of juvenile behavior with other (neuro-)toxicity tests at different life-stages

7.1 Introduction

Unfortunately, many complex behaviors that may serve to directly interpret population relevant neurotoxicity effects only develop later in the fish's lives, resulting in a higher demand for animal testing (Colwill and Creton, 2011). In contrast, the alternative methods that are constantly being developed to improve animal welfare (or surpass the need for animal testing altogether) as well as to allow more rapid assessments of unknown substances are often abstract and difficult to relate. Therefore, the different methods need to be evaluated based on their sensitivity and predictive capacity before one may replace another. I this section, I correlate early larval swimming ability with shoal-wide juvenile behavior assays (results from chapter 5 & 6), histopathological analysis of juvenile zebrafish and the short-term reproduction rate of young adults.

My model pollutants for this part were once again fluoxetine (FLX), carbamazepine (CBZ), paraoxon methyl (PXM) and tris-(1,3-dichloroisopropyl) phosphate TDCPP for the same reasons as outlined in chapter 6. TDCPP, however, was not used for additional animal experimentation due to the availability of published works and its weak effect during my previous experiments, but histological samples from previous tests with this substance could still be evaluated.

By including this variety of mechanisms, I aim for a broad view of the related effects and expect to detect some effects that may be missed in a single substance study. On the other hand, a method that is less sensitive than the others for all these examples may be assumed to be less sensitive for the overall majority of substances and will not need to be considered in a future test battery.

7.2 Materials and methods

For details on fish maintenance, please refer to the "basic methods" section.

Experimental animals were selected and randomly divided into treatment groups at different life stages for each assay: embryo to larva (0-6 dpf; larval motility), juvenile (21-34 dpf; histopathology) or adult (approx. 6 months; fecundity). Until these respective onsets of exposition, all fish were treated the same way as non-experimental cohorts in the breeding facility.

7.2.1 Larval motility assay – egg selection, exposure & observation

The larval motility assay was mostly based upon the Fish Embryo Toxicity (FET) test (test guideline 236; OECD, 2013a). Essentially, the standard 96-hour procedure was extended by two days and fish were placed into larger than usual volumes (3.5 mL) to allow observations of coordinated swimming behavior in addition to teratogenic effects.

Each 12-well plate (TPP, Trasadingen, Switzerland) was prepared with ten wells holding a test chemical dilution (see Table 3) and two internal negative controls (IC). 7 plates with different exposure concentrations and an entire negative control plate (NC) made up each replicate experiment. In the case of CBZ, 0.5 % dimethyl sulfoxide (DMSO) was added as solvent so the corresponding NC plates also contained dilution water + 0.5 % DMSO to act as solvent control. As many chemicals adsorb to plastic, the well plates were pre-saturated with the respective solutions and incubated at 26 °C for about 24 h prior to egg selection. Test solutions were exchanged immediately before the eggs were introduced. Once again, the maximum exposure concentrations were based on ≥ 96 h EC_{10} values from the literature and own preliminary experiments to avoid acute toxic effects.

Table 3: Model substances for larval motility assays and the corresponding test concentrations.

Fluoxetine (µg/L)	1.25	2.5	5	10	20	40	80
Paraoxon-methyl (mg/L)	0.03125	0.0625	0.125	0.25	0.5	1	2
Carbamazepine (mg/L)	0.625	1.25	2.5	5	10	20	40

Within an hour after dawn, eggs were collected from the spawning tanks and only roughly assessed for exposure to begin as early as possible. Small batches of randomly selected fertilized eggs were pre-exposed at the 4 to 32 cell stage in 60 mm glass crystallization dishes that contained 10 mL of the respective chemical solution (see below). During closer inspection under a stereomicroscope, eggs with visible defects (e.g., membrane damage, malformed cells or vesicle formation) were discarded. The remaining embryos were individually placed in 12-well plates. Once fully stocked, plates were sealed with self-adhesive film (SealPlate®; Excel Scientific, USA) kept at 26 ± 0.5 °C and a light/dark cycle of 14/10 h in a KB115 incubator (Binder, Germany).

During the exposure period, subjects were microscopically monitored every day. Any fish with overt sublethal effects was immediately removed and euthanized by means of 96 % ethanol (0-4 dpf) or 400 mg/L tricaine (5-6 dpf). Tricaine was also used to euthanize all larvae after the experiments were completed. Test solutions were exchanged daily to maintain good water quality and constant exposure.

At 6 dpf, larvae were fed with *Paramecium caudatum* (own culture) and the plates were placed into a DanioVision Observation Chamber (Noldus, Wageningen, the Netherlands) in random succession. After approx. 5 minutes of acclimation under visible light, each plate was filmed with the integrated IR camera under visible light for 10 minutes and in the dark for another 10 minutes.

Water temperature was maintained at 26 °C using a Temperature Control Unit (Noldus). Pylon Viewer 4.2.1.4845 (Basler, Ahrensburg, Germany) was used to control the acquisition parameters (25 fps, 1288x1026 px) and to make fine adjustments to the image quality. The movement in each well was tracked with EthoVisionXT 11.5 (Noldus).

EthoVisionXT also served to evaluate swimming patterns and the general data quality. All data were exported as spreadsheets and processed with SigmaPlot 14.0 (Systat Jandel, Erkrath; Inpixon, Düsseldorf, Germany) for statistical analysis by means of one-way ANOVA and Dunn's *post-hoc* test.

7.2.2 Preparation and analysis of histological sections

Fish samples were collected after independent flow-through exposure experiments with observable behavior effects: details on rearing and the previous treatment of these fish with my model substances are described in chapters 5 & 6.
Immediately following those juvenile behavior tests, the 5-week-old fish had been euthanized in ice cold 400 mg/L tricaine (MS-222) solution and stored in modified Davidson's medium (Latendresse et al., 2002) in a standard refrigerator.
Three to 18 months later, the zebrafish were removed from the fixative and infiltrated with Leica Surgipath Paraplast Plus by means of a Leica TP1020 semi-enclosed automatic tissue processor (both: Leica Biosystems, Wetzlar, Germany). This machine cycled the samples through different dilutions of ethanol, isopropanol, xylene, and finally paraffin for dehydration and infiltration.

The fish were then embedded in paraffin using a Leica EG 1140 H embedding station (Leica Biosystems). The fish were placed with their ventral sides towards the cutting surface, as to be cut from the anterior to the posterior end, before they were embedded. Three fish from the same tank were placed in each block and cooled on a Leica EG 1140 C cooling plate until solid (Leica Biosystems). The embedded samples were kept in a refrigerated room at 8°C until cutting and staining.

For 1-2 days before sectioning, the samples were kept in a freezer at -18°C to improve the consistency of the cuts. The sections were cut 4 µm thick using a Thermo Scientific Shandon Finesse ME microtome (Thermo Fischer Scientific, Gothenburg, Sweden). The sliced sections were carefully transferred to a heated water bath (32°C) with a moist brush to allow the segments to stretch out, facilitating transfer and adhesion to the glass slides. Once they were placed on glass, the sections were protected from dust and dried at room temperature for 2-3 days. The sections were then stained using a Tharmac Cellstain 15 (Tharmac Laboratory Solutions, Wiesbaden, Germany). Before staining, the sections were incubated at 40°C for 20 minutes. As outlined in Table 4, the paraffin was removed using the xylol substitute "X-TRA-SOLV" (Medite Medical, Burgdorf, Germany). Then, the slides were rehydrated in isopropanol and a descending series of ethanol dilutions and stained with Mayer's hematoxylin (nuclei, purple) and erythrosine (positively charged plasma proteins, red). Finally, the sections were rehydrated in an ascending series of ethanol dilutions and isopropanol. After staining, the slides were sealed with "X-TRA-KITT" (Tharmac Laboratory Solutions) and left to dry for 3-5 days.

Microscopic analysis was carried out with a Leitz Aristoplan microscope (Leica Biosystems), an Imaging Source camera (The Imaging Source Europe, Bremen, Germany), and NIS Elements software (Nikon Instruments, Tokyo, Japan). The images were processed using FIJI/ ImageJ (Schindelin et al., 2012). The same software was used to reduce background spots and noise. Adobe Lightroom 8 (Adobe, San Jose, USA) was used for white balance adjustments. For quantitative analysis of the retina, an average of 20 measurements of the inner plexiform layer (IPL) were taken in the plane of the optic nerve. The resulting mean IPL thickness was normalized using the overall retinal thickness at the entry point of the optical nerve to repeatably account for individual body size variation.

7.2.3 Short term reproduction assay

I finally carried out a fish short term reproduction assay (FSTRA) based on OECD TG 229 (OECD, 2012) to test whether the prominently altered behavior in earlier FLX experiments (chapters 5 & 6) also correlates to an impact on fecundity as a vital aspect of population fitness.

To this end, 10 L glass tanks were set up in a similar manner as described earlier (chapter 5), with rotameters (Yokogawa, Ratingen, Germany) supplying fresh water at a rate of 1.2 L/h and peristaltic pumps (Gilson, Limburg an der Lahn, Germany) adding the appropriate stock solutions at 30 mL/h. The resulting flow-through rate was approx. 3 volume exchanges per day and the water quality was regularly monitored. Constant low-pressure aeration ensured sufficient concentrations of dissolved oxygen and the temperature was kept at 26 ± 1.5 °C. Visible accumulations of debris were promptly extracted from the tanks.

6-month-old adult zebrafish were randomly selected and exposed to the test substance in groups of 10 (5 females, 5 males) for 21 days after 20 days of habituation to the exposure setup in clean water (pre-exposure). Males were identified by the yellow hue of their sides and anal fin and were paired with females of different ancestry. They were fed twice a day, once with commercial flake food (“TetraMin”; Tetra, Melle, Germany) and once with *Artemia* nauplii (48-hour incubation; Sanders, Ogden, Utah, USA).

Three independent experiments were run in parallel, each consisting of a dilution water control and three different concentrations of FLX (20, 40, 80 µg/L). The position of each treatment within the tank array was randomized. A slanted glass tray covered with stainless steel mesh was placed into each tank every afternoon and topped at the shallow end with a string of green glass beads as spawning stimulus. In the morning, all trays were carefully removed and inspected for viable eggs 2 hours after sunrise. After the egg count, they were rinsed and scrubbed with ethanol and deionized water. Engraved markings ensured each tray was returned to the same exposure tank every day.

Immediately after the final egg collection on the 21st day of exposure, the fish were euthanized by submersion in 400 mg/L tricaine (MS-222) on ice.

Quantitative fecundity was recorded as the number of fertilized eggs and evaluated daily and cumulatively. Using SigmaPlot 13.0 (Systat Software, Erkrath, Germany), I applied one-way ANOVA and Dunnett’s *post-hoc* test to determine statistically significant differences between the treatment groups as well as the acclimation and exposure phases.

7.3 Results

7.3.1 Larval motility was reduced after exposure to model substances

By the time locomotor activity was to be measured on the sixth day of exposure, the fish that were exposed to ≥ 1.25 µg/L FLX frequently showed reduced pigmentation but no other teratogenic effects. I also saw a baseline mortality around this time independent of the substances and concentrations, probably linked to the first critical phase of larval survival: filling the swim bladder, which usually occurs between 5 and 6 dpf. The average death rate was 8.8 ± 10 % (data not shown), including moribund individuals with clearly uninflated swim bladders that were removed once identified.

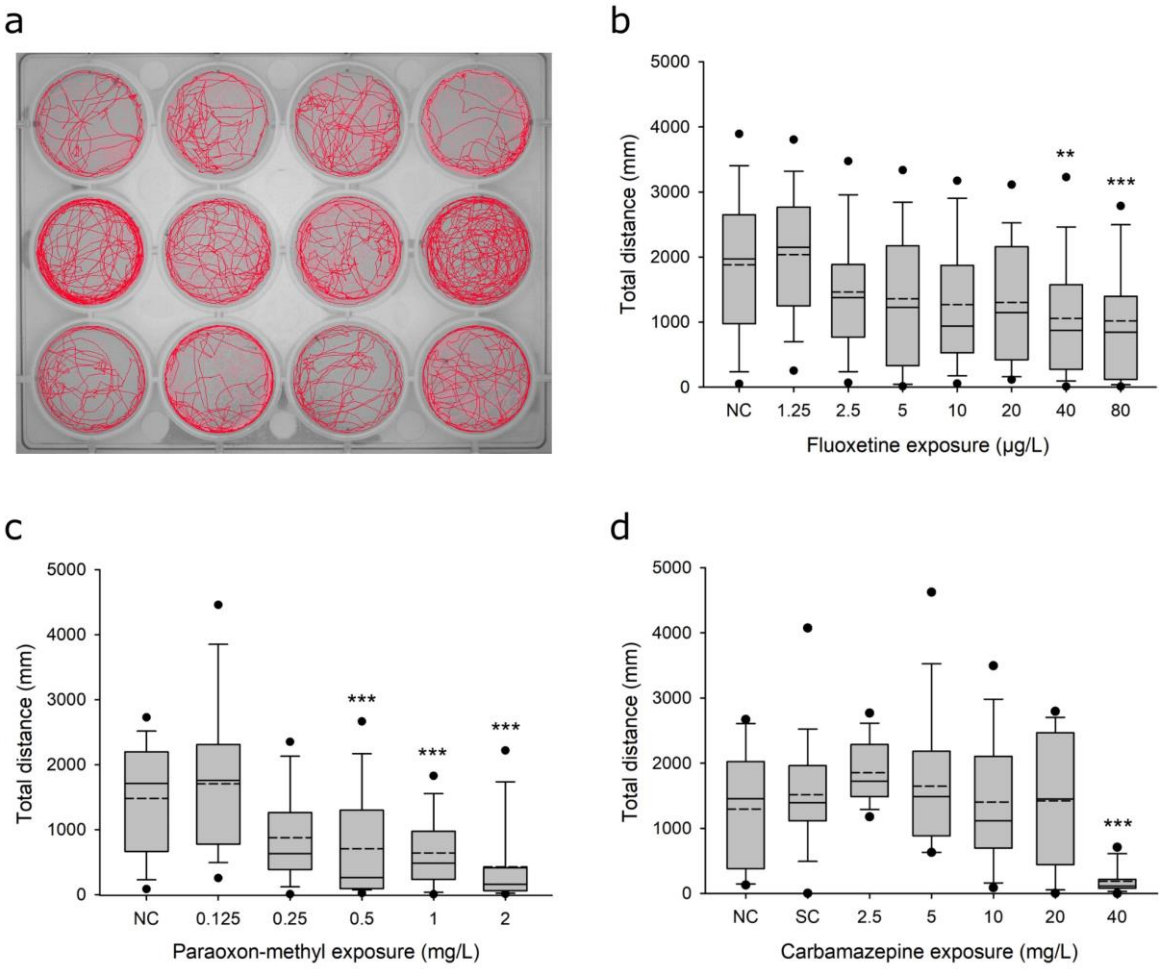

Fig. 17: Effects of continuous exposure to different substances on the locomotor activity of 6-day-old zebrafish (Danio rerio) larvae. a) The animals were filmed for 10 minutes under visible light while their movement was tracked (red) and the total swimming distance was calculated by an automated system. b) Effect of 1.25 to 80 µg/L fluoxetine compared to a negative control group (NC). c) Effect of 0.125 to 2 mg/L paraoxon-methyl with corresponding negative control. d) Effect of 2.5 to 40 mg/L carbamazepine compared to the corresponding negative control and a solvent control (SC) exposed to 0.5 % DMSO. Significance of differences from controls by one-way ANOVA-on-ranks in combination with Dunn's *post-hoc* test: ** $p < 0.01$, *** $p < 0.001$. Data produced jointly with Lisa Stötzel.

The locomotor activity was initially assessed in different ways – total distance, average velocity and proportion of activity to resting periods – which were all closely correlated (not shown). Of these, the total swimming distance appears to be most robust against the influence of skipped frames or slight tracking gaps in the analysis software and relates well to my question of swimming ability, so I chose this metric going forward.

Compared to the negative control, FLX treatment of ≥ 40 µg/L caused a significant decrease in the total distance traveled during 10-minute trials, with the median distances covered by the control group (1974 mm) being more than twice as high as the treated larvae (873 and 846 mm; Fig. 17). Likewise, PXM was significantly effective at concentrations of 0.5 mg/L and above and the ratio of control to exposure group distances was even higher (medians: 1713 mm and 158 mm for the control and 2 mg/L PXM, respectively). Larvae exposed to 40 mg/L CBZ also covered significantly less distance ($p < 0.001$) than larvae from both control groups. Most lower trials with lower concentrations (2.5 to 10 mg/L) seem to indicate a trend towards this strong reduction, but results from the 20 mg/L groups were inconclusive and distributed much like those from controls.

There were differences between the control groups that may be explained by genetic predispositions or similar group effects. Therefore, the comparison of each trial set was limited to those controls that were run in parallel with it. Four separate trials (each including controls) were completed with FLX, three with PXM and two with CBZ.

7.3.2 Histopathology revealed no signs of acute toxicity

The fixed samples of five-week-old (35 dpf) fish from previous experiments were preserved equally well across all times of fixation (three to 18 months previously). Gonads were qualitatively assessed and appeared regular but were altogether too immature for deeper analysis. As would be expected at this age, ovary sections showed an abundance of perinucleolar oocytes and oogonia. Based on the composition of the ovarium, samples were categorized as stage 0 (undeveloped) according to the OECD guidance document for the diagnosis of endocrine-related histopathology of fish gonads (Johnson et al., 2009). The same stage was observed in all treatment and control groups. Male gonad differentiation was also observed. The absence of any recognizable spermatozoa and sparse occurrence of spermatogonia led to a classification of these samples between juvenile and stage 0.

More intuitively related to the effects I saw in the behavior tests, the main interest lay on swim bladder and eye malformations that may explain excessive surface dwelling or impaired flight reactions. Swim bladders were qualitatively assessed for apparent intactness of walls and appropriate inflation of both compartments (Fig. 18a/b). The adjacent livers helped to identify approximately equivalent cutting planes, but precise size comparisons were not attainable (nor deemed promising). There were no indications lack of inflation or damage beyond cutting artifacts and no discernible differences between any controls and treatment groups.

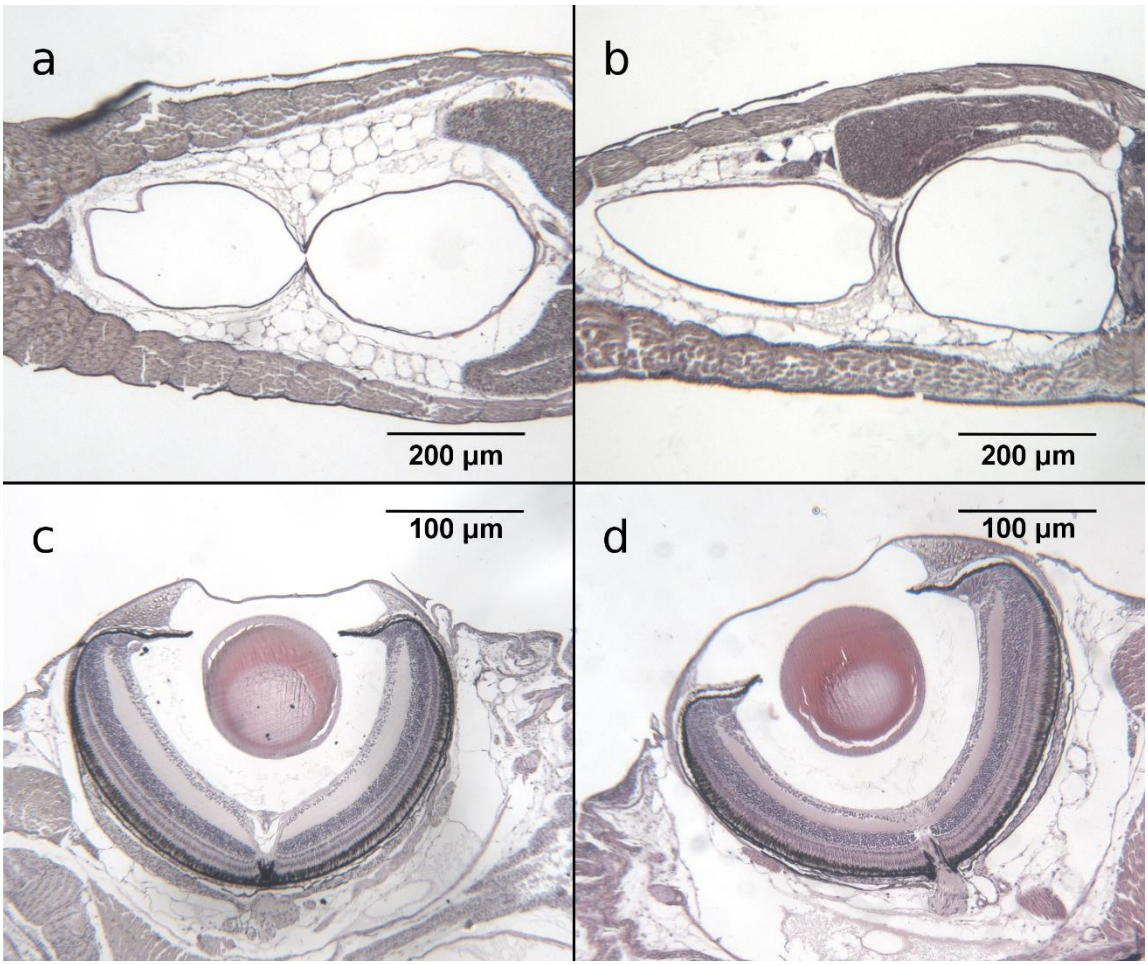

Fig. 18: Examples of histological sections of swim bladders (a, b) and eyes (c, d) of 35-day-old zebrafish (*Danio rerio*). Neither the pictured total views nor higher magnifications revealed discernible differences between fish from control groups (a, c) and those treated with 40 µg/L fluoxetine for the previous 14 days (b, d). Stained with hematoxylin (purple) and erythrosine (red). Data produced jointly with Marius Schubert.

The qualitative assessment of retinal sections revealed no visible damage in the zebrafish exposed to up to 80 µg/L FLX, 0.5 mg/L PXM, 0.96 mg/L TDCPP or 10 mg/L CBZ. Plexiform and nuclear layers of the retina showed no observable difference between treated and control zebrafish besides natural variations (see Fig. 18c/d for a representative example).
The thickness of the inner plexiform layer (IPL) particularly lends itself to quantitative analysis and was measured at 20 different points per sample. Although there were apparent differences between some entirely independent trials at first glance (up to 13 %), the deviations in the mean thickness for each exposure group compared to the corresponding controls were hardly detectable (Fig. 19b). I believe those apparent differences are related to genetic factors that are negated by comparing only the corresponding groups, i.e., each exposure group's inherent predisposition for thinner or thicker IPL is balanced by the same trend in its sibling control group for better comparability across trials.

None of the tested substances had a significant effect on the IPL thickness at the concentrations used herein but there was a noticeable trend towards an increase after TDCPP and CBZ exposure (highest median deviation from the controls: CBZ, + 3.3 %).

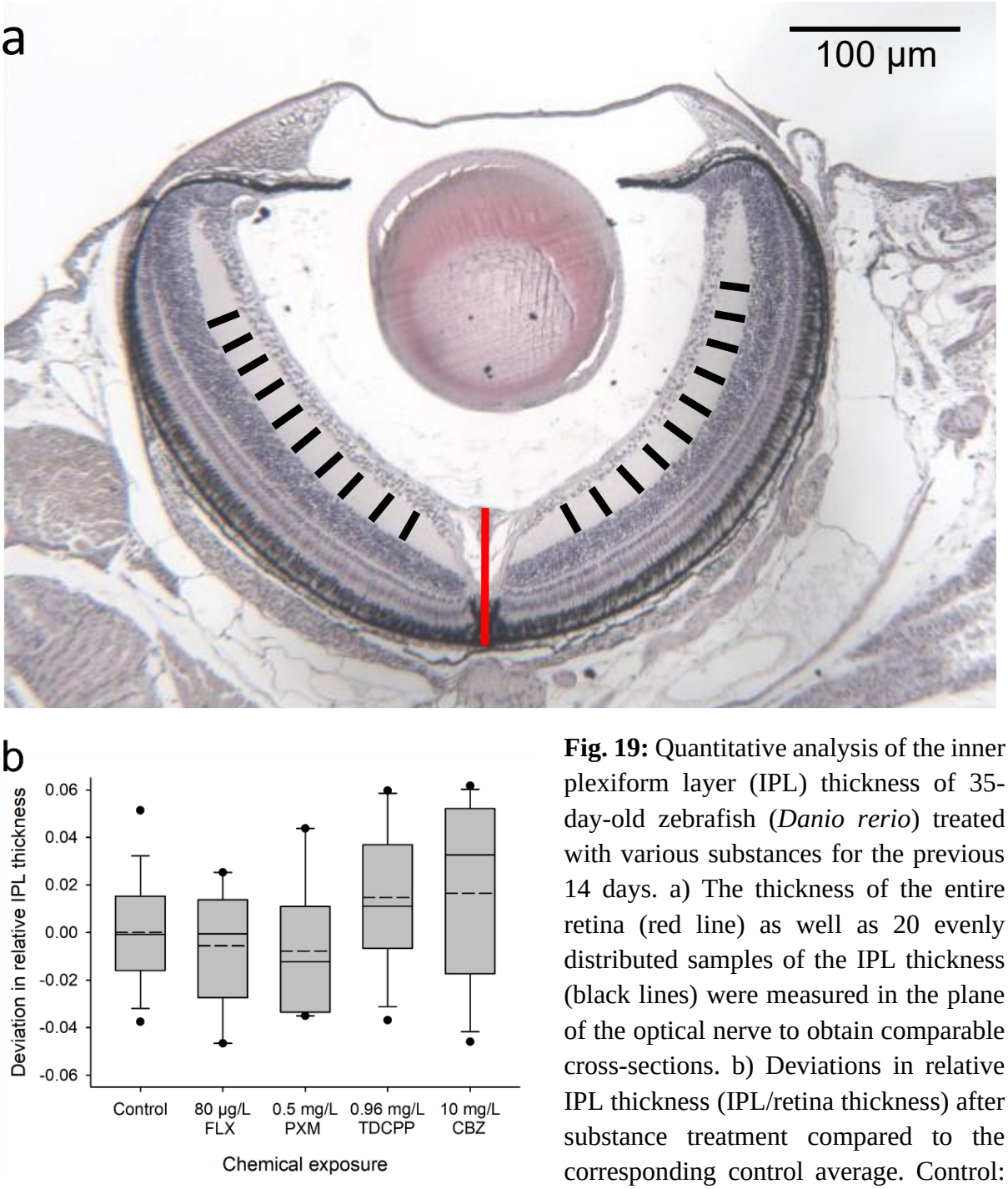

Fig. 19: Quantitative analysis of the inner plexiform layer (IPL) thickness of 35-day-old zebrafish (*Danio rerio*) treated with various substances for the previous 14 days. a) The thickness of the entire retina (red line) as well as 20 evenly distributed samples of the IPL thickness (black lines) were measured in the plane of the optical nerve to obtain comparable cross-sections. b) Deviations in relative IPL thickness (IPL/retina thickness) after substance treatment compared to the corresponding control average. Control: n=36; FLX: fluoxetine, n=5: PXM: paraoxon-methyl, n=7; TDCPP: Tris(1,3-dichloroisopropyl) phosphate, n=11; CBZ: carbamazepine, n=11. Data produced jointly with Marius Schubert.

7.3.3 Fluoxetine diminishes zebrafish fecundity

All groups began to produce fertilized eggs shortly after the onset of the acclimation period and continued to do so in a regular manner until exposure began on day 0. Thereafter, egg production dropped in a concentration dependent fashion and clear differences in the cumulative number of eggs were seen as early as five days into the experiment (Fig. 20a). By the end of the assay (day 20), the control groups had laid more than three times as many viable eggs as the fish exposed to 40 and 80 µg/L FLX and 1.5 times as many as the 20 µg/L FLX groups.

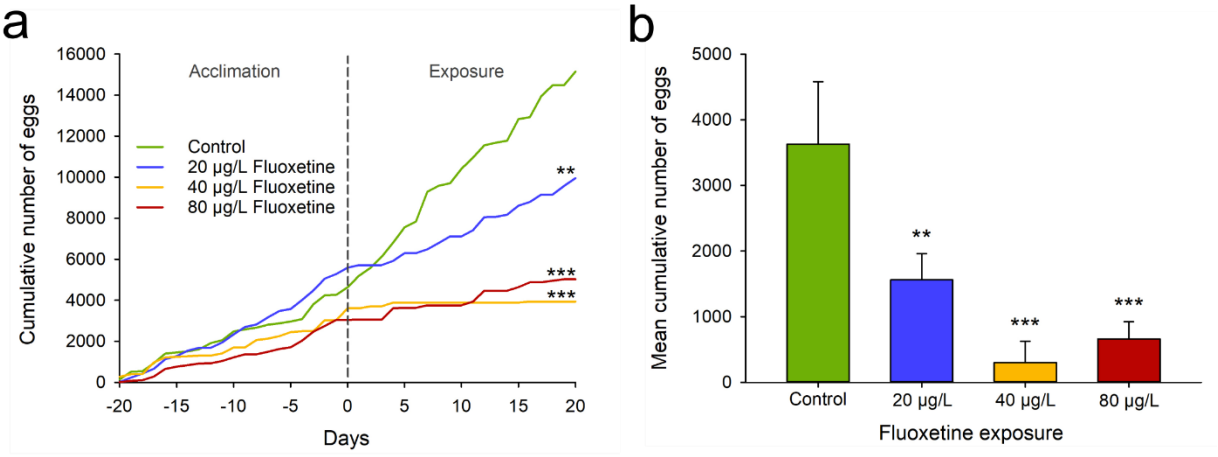

Fig. 20: Number of fertilized eggs laid by six-month-old zebrafish (*Danio rerio*) during a 20-day fish short-term reproductive assay under fluoxetine exposure. a) Cumulative number of fertilized eggs laid from 20 days before until 20 days after the onset of exposure to 20 to 80 µg/L fluoxetine. b) Mean cumulative number of eggs laid by each of three replicate groups during the exposure period. Significance of differences from the control by one-way ANOVA-on-ranks in combination with Dunnett's *post-hoc* test: ** $p < 0.01$, *** $p < 0.001$.

The mean cumulative number of eggs per replicate during the exposure phase followed a very similar pattern and confirmed the high statistical significance of the differences between the treatment groups (Fig. 20b). On average, control groups laid more than twice as many eggs as those exposed to 20 µg/L FLX and over five times the amount obtained from the 80 µg/L groups. Exposure to 40 µg/L had an even stronger impact in this study, with one group failing to lay any eggs during the exposure phase.

This reduced egg output may in part be explained by a strongly increased failure rate (days without any viable eggs) after FLX exposure (Fig. 21). Failures appeared to become more frequent during the exposure phase in all treatment groups and were significantly more common than in the controls after 40 to 80 µg/L FLX exposure ($p < 0.001$ and $p < 0.01$, respectively).

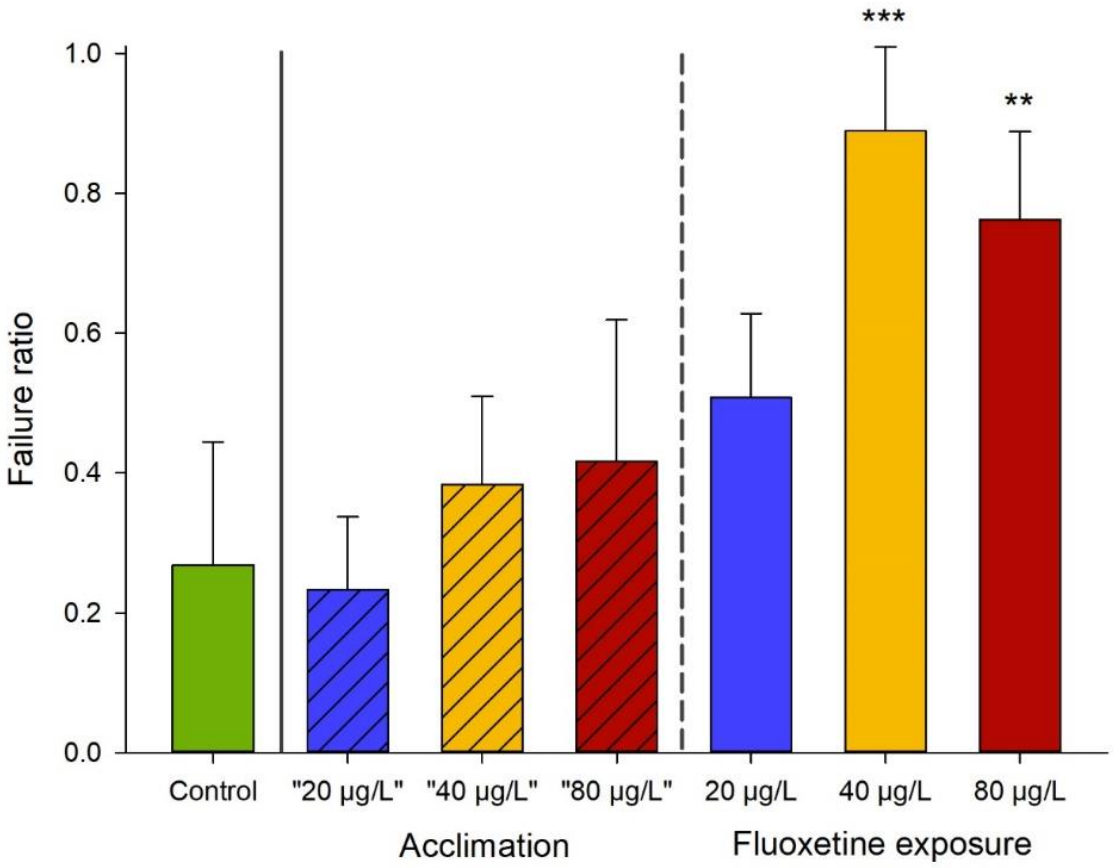

Fig. 21: Comparison of the failure rate (portion of days without a single fertilized egg) during 20 days of acclimation and the following fluoxetine exposure of the same duration. Significance of differences from the control by one-way ANOVA-on-ranks in combination with Dunnett's *post-hoc* test: ** $p < 0.01$, *** $p < 0.001$.

The 40 µg/L FLX failure rate was also significantly different from the same group's pre-exposure failure rate (Fig. 21, yellow bars). The 80 µg/L groups also showed a sharp increase compared to their previous average, but this was not significant according to Dunnett's test ($p < 0.069$).

7.4 Discussion

Out of the three methods applied to my model substances, only histopathology failed to produce tangible effects at the tested concentrations (up to 10 mg/L CBZ, 80 µg/L FLX, 0.5 mg/L PXM and 0.96 mg/L TDCPP). There was, however, a benefit from the observations of healthy organs as well: they showed that the fish should be physiologically capable of normal behavior, so the reason for the previously observed changes (chapters 5 & 6) indeed appears to be neurotoxicity. This result shows yet again how behaviors can be more sensitive than classic endpoints due to their complex and integrative nature. Similarly, Beker van Woudenberg et al. (2014) found CBZ to be effective on growth retardation and behavior well below a concentration that caused histologically observable brain defects.

The model substances did cause significant alterations of larval motility at least at the highest concentrations tested, but even an absence of effects may not be interpreted to indicate an absence of population relevant impairments to sensory organs, which are not stimulated in this highly simplified assay. In fact, it has been shown that early sensing is more important for larval escape reactions than speed or direction (Nair et al., 2017), so more detailed behavior tests are warranted after an inconspicuous larval motility assay.

FSTRA, the most extensive of the attempted experiments, was only carried out with FLX since its effective concentrations between previous tests had varied rather strongly and it was so apparent that specific neurotoxicity was involved. Despite the absence of gonad damage in the histological assessment, FLX clearly reduced the fecundity of fish treated with concentrations far below the FET EC_{10}. The seemingly stronger effect of the medium concentration (40 µg/L) might be explained by individuality: if a dominant female is particularly afflicted by changes preventing it from reproducing, it may still hinder the entire rest of the group and cause an increased number of complete failures. This seems to have happened more often in the 40 µg/L groups than others (Fig. 21).

Instead of further (seemingly) independent discussion of this chapter, I shall conclude with a final look at the entirety of my findings in context.

8. Conclusions

8.1 Juvenile behavior put into perspective: A comparison of methods

To compare timing, effort, and sensitivity of various neurotoxicity tests with zebrafish, I compiled my own findings as well as the available literature into a comprehensive overview (Table 4). If several plausible studies with similar results were found, I listed the highest reported sensitivity within the widely supported order of magnitude. In contrast, if a single publication gave exceptionally low supposedly effective concentrations, its assumptions and methodology were critically evaluated and only plausible results were included. Studies with similar substances (e.g. paraoxon-ethyl) or different fish species were also not included in this summary.

Table 4: Sensitivity of various methods using zebrafish (Danio rerio) and my chosen substances. Unless otherwise noted, concentrations are lowest observed effect concentrations (LOEC). Findings from the present dissertation are printed in boldface. Grey fields indicate no comparable effective concentrations could be found.

Method	Carbamazepine	Fluoxetine	Paraoxon-methyl	TDCPP
Spontaneous tail coiling (24 h)	EC_{10}: 24.5 mg/L (Ogungbemi et al., 2020)	LOEC: > 12 mg/L† (Zindler et al., 2019b)	EC_{10}: 0.2 mg/L (Ogungbemi et al., 2020)	LOEC: 0.3 mg/L (Cheng et al., 2017)
FET (96 h, EC_{10})	41 mg/L (Beker van Woudenberg et al., 2014)	7.39 mg/L (Zindler et al., 2019b)	2 mg/L (Kais et al., 2015)	≈ 0.86 mg/L (McGee et al., 2012)
Larval motility (6 dpf)	**40 mg/L** (chapter 7)	**40 µg/L** (chapter 7)	**0.5 mg/L** (chapter 7)	≈ 2 mg/L (Dishaw et al., 2014a)
Juvenile novel tank test (35 dpf)	**10 mg/L** (chapter 6)	**≤ 5 µg/L** (chapter 5)	**0.5 mg/L** (chapter 6)	**> 0.96 mg/L†** (chapter 6)
Juvenile predator response (35 dpf)	**10 mg/L** (chapter 6)	**40 µg/L** (chapter 5)	**0.5 mg/L** (chapter 6)	**0.96 mg/L** (chapter 6)
Fecundity [duration]	5 µg/L [48 d] (Galus et al., 2013)	**20 µg/L [21 d]** (chapter 7)	no data available	20 µg/L [6 mo] (Wang et al., 2015)
Novel tank test (adult)	3 mg/kg (*oral dosing*) (Kulkarni et al., 2014)	≤ 100 µg/L (Cachat et al., 2011)	no data available	0.13 mg/L (Oliveri et al., 2015)

† No effect found up to this concentration; no further testing.

Long-running fecundity tests with adult zebrafish were among the particularly sensitive methods (earliest detection of CBZ and TDCPP effects) but are also the most problematic from an animal welfare standpoint and do not allow deductions about neurotoxic effects as they may integrate a wide array of fitness impairments. In the case of CBZ, all other assessed methods were three orders of magnitude less sensitive than fecundity over 48 days of exposure (5 µg/L; Galus et al., 2013) but rather similar to one another (10 to 41 mg/L; Beker van Woudenberg et al., 2014, Ogungbemi et al., 2020). It should be noted that the only reported effective CBZ concentration in adult novel tank tests (Kulkarni et al., 2014) cannot be readily compared with the remaining values due to different methodology (oral dosing instead of exposure through the surrounding water). TDCPP effects, on the other hand, have been detected in adult novel tank tests with only 6-fold reduced sensitivity (0.13 mg/L; Oliveri et al., 2015) compared to a six-month fecundity test (20 µg/L; Wang et al., 2015).

The lowest detection limit for FLX effects was achieved with the juvenile novel tank assay described in chapter 5, with the lowest tested concentration (5 µg/L) still causing significant effects. My 21-day fecundity tests were at least four times less sensitive (20 µg/L), similar to larval motility and juvenile predator response assays (40 µg/L). PXM effects were best detected with spontaneous coiling (0.2 mg/L; Ogungbemi et al., 2020), larval motility or juvenile behavior assays (0.5 mg/L).

It should be noted that although some publications give even lower values for certain method/substance combinations, I excluded those from my overview that lack certainty or comparability. For instance, Qiang et al. (2016) showed an effect of CBZ on coiling at a concentration as low as 5 µg/L – approx. 5000fold lower than Ogungbemi et al. (2020) – but their fish were kept in constant darkness and several higher concentrations were ineffective in the same study. Similarly, there is a single new study demonstrating effects of FLX in the adult novel tank test at ng/L concentrations, but they found no statistical significance and the observation was opposite to the widely demonstrated anxiolytic effect of the substance (Orozco-Hernandez et al., 2022). In the case of larval motility as assessed by Dishaw et al. (2014a) I condensed the two effective TDCPP concentrations (dark or light conditions) into one intermediate value for improved readability.

Overall, the recently proposed juvenile novel tank test appears to be a good compromise with a high sensitivity for most substances while directly analyzing a population-relevant endpoint. As noted in chapter 6, an adaptation of the exposure period may also improve detection of effects from cell stage specific toxins like TDCPP. Spontaneous coiling is more abstract, but very sensitive except towards substances with a later onset of neurotoxicity (FLX). Its main advantage is, of course, that it provides a very early alternative to animal testing. The substances that had an effect on coiling also caused population relevant behavior changes at similar concentrations later in life, supporting the interpretation of this endpoint as a valid early warning system.

8.2 Recommendations for routine use of behavior tests in ecotoxicology

Embryonic tail coiling and larval motility appear to be affected at the same concentration range as population-relevant behavior patterns later in life. Long-term fecundity tests may be even more sensitive, but the connection to neurotoxicity is vague and they are too costly in terms of both work and animals to be a part of an efficient screening battery.

To reduce animal testing as far as possible, I propose to carry out 24 h coiling assays at concentrations up to the 96 h EC_{10} as determined by FET. This should be followed by a larval motility assay if the results are inconclusive. Only if there is no effect on coiling or larval motility, it is worth the animal sacrifice and effort to conclude the investigation with a juvenile novel tank test (Fig. 22).

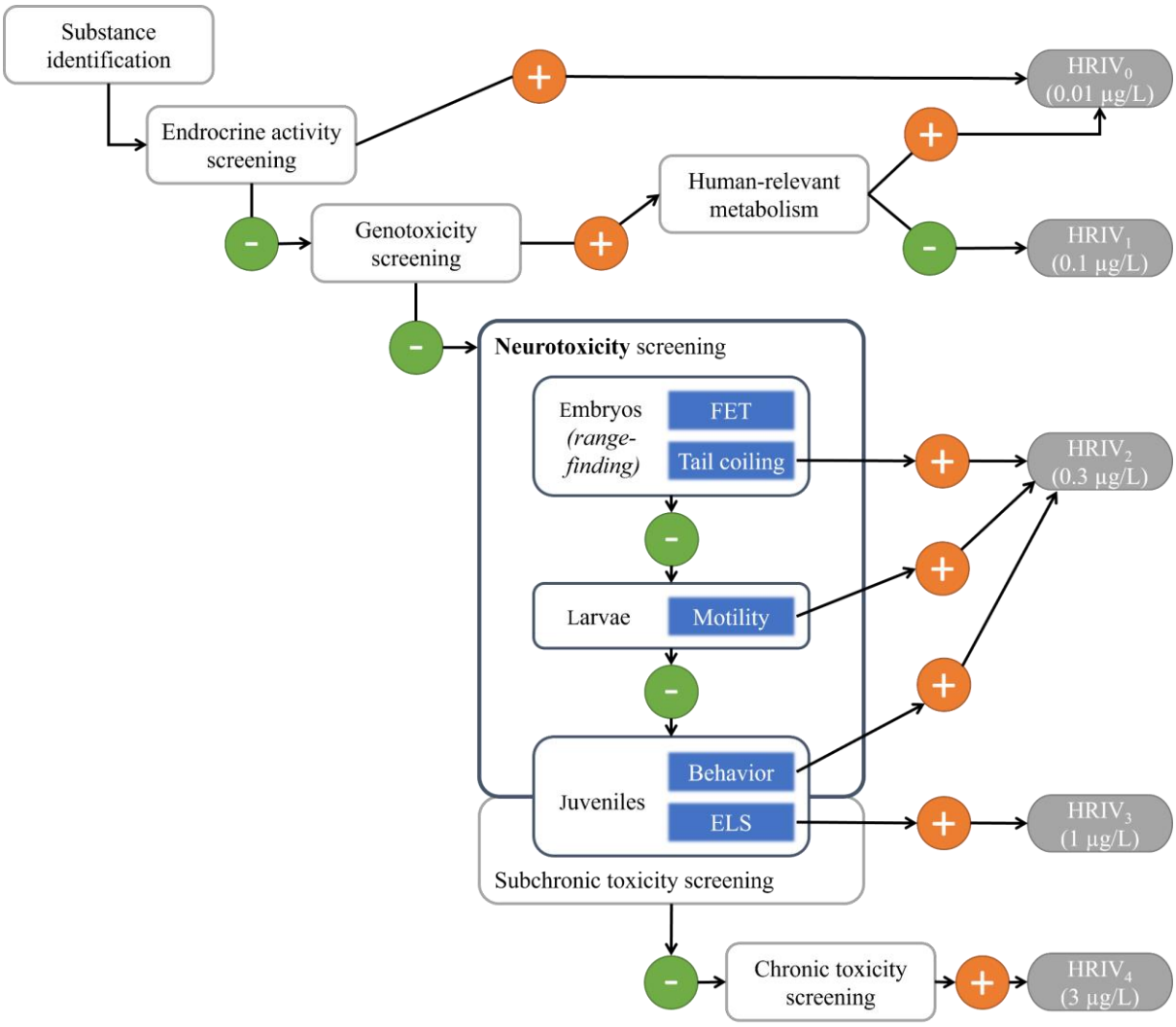

Fig. 22: Recommended sequence of *in vivo* neurotoxicity testing with zebrafish at different life-stages. HRIV = health related indication value. "+" indicates specific effects have been found or data is missing, "-" indicates that tests detected no effect. Immunotoxicity (same HRIV tier as neurotoxicity) is omitted for the sake of clarity.

While *in vivo* testing still seems indispensable for neurotoxicity in fish at this point, the proportion of studies that could be stopped after clear findings during embryonic testing and cases where effects differ significantly between embryos and later life stages should be monitored closely in order to evaluate the necessity of juvenile testing on a regular basis.

For regulatory purposes, a sufficient additional safety factor should be applied to the experimentally effective concentrations from this reduced approach until there is reliable data about a given substance's long-term effects in the environment. The long-term fecundity impact of my model compounds (where available) indicates this factor should be at least 1000. The previously defined $HRIV_2$ (0.3 µg/L, cf. Figs. 1 & 22) fulfills this suggestion for most of my model substances as they were effective at concentrations above 0.3 mg/L (0.3 µg/L×1000). In case of fluoxetine (effective down to at least 5 µg/L in juvenile behavior tests), the limit should be 5 ng/L.

8.3 Environmental relevance of the determined effective concentrations

PXM has been found in the environment at concentrations around 1 µg/L, i.e. two orders of magnitude below the spontaneous coiling EC_{10} at 0.2 mg/L (Ogungbemi et al., 2020, Papadopoulou-Mourkidou et al., 2004).

The other model substances, however, have been detected at disconcerting concentrations in certain places: Ternes (1998) found up to 6.3 µg/L CBZ in waste water treatment plant (WWTP) effluents and up to 1.1 µg/L in German rivers which may be sufficient to decrease fish fecundity according to Galus et al. (2013; LOEC: 5 µg/L). Likewise, the FLX concentration of $\leq$ 5 µg/L which caused notable effects in juvenile novel tank tests (chapters 5 & 6) is worryingly close to the high ng/L values found in WWTP effluents as well as freshwater in Northern America (Metcalfe et al., 2003, Mole and Brooks, 2019). Finally, the high persistence of TDCPP has led to local accumulation as high as 56 µg/L in Chinese sewage water, more than twice the concentration reported to reduce fish fecundity over 6 months (Wang et al., 2020). Surface water concentrations are not far behind, reaching 1.4 µg/L near Californian WWTPs for example (Maruya et al., 2016).

The detection of neurotoxic effects at or near these environmental concentrations is certainly an immediate cause for concern and warrants further and more detailed testing, especially since prolonged exposure may prove even more harmful than the limited timespans associated with most laboratory experiments (Pfluger and Dietrich, 2001).

8.4 Perspectives

8.4.1 Automation is highly desirable

As outlined in chapter 6, the accuracy of the juvenile behavior tests may be vastly improved by automated tracking. Although there has been a lot of progress on the topic of tracking multiple subjects in a common arena, current solutions still require larger fish and/or the marking of individuals which may in itself alter the behavior of the affected individual as well as its shoal mates (Miller and Gerlai, 2007). Besides such optical effects, fish may metabolically react to the injected dyes in addition to the toxicants one wants to assess in the first place, so this is not an option in ecotoxicological studies.

Once suitable software does become available, it should be integrated into the protocol as soon as possible and may perhaps even be used to re-analyze existing recordings to gain new insights without repeating the experiments themselves. Besides result quality, the throughput rate of the either assay would of course be considerably increased by truly automatable means of tracking.

8.4.2 Additional endpoints in behavior testing

Apart from fecundity, I have mainly assessed behaviors that influence the chance of individual survival under threat of predation. If the method is to be augmented further, several other population relevant endpoints may also be highly informative:

Feeding has a direct impact on individual fitness and ultimately reproduction. Particularly the success rate when hunting live prey depends on healthy behavior and may be affected by subtle neurotoxic impairments. The main challenge is finding a repeatable way to measure hunting efficiency early in life that does not interfere with the chemical treatment (as dyed prey animals could) and is ideally compatible with the already described methods.

Another potentially interesting aspect of population fitness is group preference, i.e., how readily shoals form under new conditions and whether isolated fish strive to become part of a safer group. This has been investigated by several authors using mirrors, screens or compartmentalized choice tanks that allow groups of fish and/or single fish to swim towards or away from each other (Ansai et al., 2016, Blaser and Gerlai, 2006, Fernandes and Gerlai, 2009). The associated paradigms require more space than the methods I have described, but if the observation tank is elongated for improved predator response assays it may also offer enough room to observe social behavior. The efficient switch between experimental “modes” during a group’s testing phase may be an issue, but certainly not impossible to overcome.

8.4.3 Exposure durations and integration of juvenile behavior into standard procedures

As outlined earlier, beginning the exposure phase of the juvenile behavior studies at 21 dpf is beneficial from animal welfare and practical standpoints. The other suggested methods (embryonic and larval behavior) should be capable of detecting most early-onset effects that would be missed at later stages. However, a gap remains and is not to be neglected: substances that are most effective between 6 and 21 days of development, or cause effects during the first

6 days that only become evident through more complex behaviors later in life, will not yet cause deviations in coiling or larval motility but juveniles exposed after the ideal window may develop normally. It should therefore be attempted to prepone the onset of exposure by one week (14 dpf) to reduce this problem. An even earlier onset would be desirable in order to achieve a proper chronic exposure period of at least 28 days (Ahlers et al., 2006).

The newly proposed combination with embryonic and larval assessments may allow a sequential approach: the same fish (exposed from few hours after fertilization) could be assessed as embryos and larvae on well plates, then raised in small tanks with the respective exposure concentrations until 14 dpf and then split into the final shoals. Thereby, the total number of experimental animals could be reduced although the remaining ones would be distressed for a longer time and a higher number than ultimately needed would be raised until 14 dpf to make sure there are enough survivors beyond the critical point of swim bladder inflation. This trade-off should be carefully considered with animal welfare experts, of course, but there seems to be a notable potential for reducing the number of animals while speeding up the process and gaining a more substantiated understanding of population-relevant neurotoxicants.

9. Index of figures

10. References

Ahlers J., Riedhammer C., Vogliano M., Ebert R.-U., Kühne R. & Schüürmann G. 2006. Acute to chronic ratios in aquatic toxicity – variation across trophic levels and relationship with chemical structure. *Environmental Toxicology and Chemistry,* 25**,** 2937-2945.

Ahmed O., Seguin D. & Gerlai R. 2011. An automated predator avoidance task in zebrafish. *Behavioural Brain Research,* 216**,** 166-171.

Ahmed T.S., Gerlai R. & Fernandes Y. 2012. Effects of animated images of sympatric predators and abstract shapes on fear responses in zebrafish. *Behaviour,* 149**,** 1125-1153.

Airhart M.J., Lee D.H., Wilson T.D., Miller B.E., Miller M.N. & Skalko R.G. 2007. Movement disorders and neurochemical changes in zebrafish larvae after bath exposure to fluoxetine (Prozac). *Neurotoxicology and Teratology,* 29**,** 652-64.

Ambrósio A.F., Soares-Da-Silva P., Carvalho C.M. & Carvalho A.P. 2002. Mechanisms of action of carbamazepine and its derivatives, oxcarbazepine, BIA 2-093, and BIA 2-024. *Neurochemical Research,* 27**,** 121-130.

Andersen H.R., Nielsen J.B. & Grandjean P. 2000. Toxicologic evidence of developmental neurotoxicity of environmental chemicals. *Toxicology,* 144**,** 121-127.

Ansai S., Hosokawa H., Maegawa S. & Kinoshita M. 2016. Chronic fluoxetine treatment induces anxiolytic responses and altered social behaviors in medaka, *Oryzias latipes*. *Behavioural Brain Research,* 303**,** 126-136.

ATSDR 2020. Toxicological profile for lead. U.S. Department of Health and Human Services.

Baldi I., Lebailly P., Mohammed-Brahim B., Letenneur L., Dartigues J.-F. & Brochard P. 2003. Neurodegenerative diseases and exposure to pesticides in the elderly. *American Journal of Epidemiology,* 157**,** 409-414.

Barcellos L.J.G., Ritter F., Kreutz L.C., Quevedo R.M., Da Silva L.B., Bedin A.C., Finco J. & Cericato L. 2007. Whole-body cortisol increases after direct and visual contact with a predator in zebrafish, *Danio rerio*. *Aquaculture,* 272**,** 774-778.

Barry M.J. 2013. Effects of fluoxetine on the swimming and behavioural responses of the Arabian killifish. *Ecotoxicology,* 22**,** 425-32.

Bass S.L.S. & Gerlai R. 2008. Zebrafish (*Danio rerio*) responds differentially to stimulus fish: The effects of sympatric and allopatric predators and harmless fish. *Behavioural Brain Research,* 186**,** 107-117.

Behra M., Etard C., Cousin X. & Strahle U. 2004. The use of zebrafish mutants to identify secondary target effects of acetylcholine esterase inhibitors. *Toxicological Sciences,* 77**,** 325-333.

Beker Van Woudenberg A., Snel C., Rijkmans E., De Groot D., Bouma M., Hermsen S., Piersma A., Menke A. & Wolterbeek A. 2014. Zebrafish embryotoxicity test for developmental (neuro)toxicity: Demo case of an integrated screening approach system using anti-epileptic drugs. *Reproductive Toxicology,* 49**,** 101-116.

Bencan Z., Sledge D. & Levin E.D. 2009. Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. *Pharmacology Biochemistry and Behavior,* 94**,** 75-80.

Benotti M.J., Trenholm R.A., Vanderford B.J., Holady J.C., Stanford B.D. & Snyder S.A. 2009. Pharmaceuticals and endocrine disrupting compounds in U.S. Drinking water. *Environmental Science & Technology,* 43**,** 597-603.

Best J.D. & Alderton W.K. 2008. Zebrafish: An in vivo model for the study of neurological diseases. *Neuropsychiatric Disease and Treatment,* 4**,** 567-576.

Betarbet R., Sherer T.B., Mackenzie G., Garcia-Osuna M., Panov A.V. & Greenamyre J.T. 2000. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nature Neuroscience,* 3**,** 1301-1306.

Blaser R. & Gerlai R. 2006. Behavioral phenotyping in zebrafish: Comparison of three behavioral quantification methods. *Behavior Research Methods,* 38**,** 456-469.

Braunbeck T., Böttcher M., Hollert H., Kosmehl T., Lammer E., Leist E., Rudolf M. & Seitz N. 2005. Towards an alternative for the acute fish LC_{50} test in chemical assessment: The fish embryo toxicity test goes multi-species – an update. *ALTEX-Alternatives to animal experimentation,* 22**,** 87-102.

Braunbeck T. 2009. Zebrafish embryos as a model in general toxicology. *Toxicology Letters (Shannon),* 189**,** 48-49.

Braunbeck T., Kais B., Lammer E., Otte J., Schneider K., Stengel D. & Strecker R. 2015. The fish embryo test (FET): Origin, applications, and future. *Environmental Sciences and Pollution Research,* 22**,** 16247-16261.

Brotzmann K., Wolterbeek A., Kroese D. & Braunbeck T. 2021. Neurotoxic effects in zebrafish embryos by valproic acid and nine of its analogues: The fish-mouse connection? *Archives of Toxicology,* 95**,** 641-657.

Brown R.C., Lockwood A.H. & Sonawane B.R. 2005. Neurodegenerative diseases: An overview of environmental risk factors. *Environmental Health Perspectives,* 113**,** 1250-1256.

Brustein E., Saint-Amant L., Buss R.R., Chong M., Mcdearmid J.R. & Drapeau P. 2003. Steps during the development of the zebrafish locomotor network. *Journal of Physiology (Paris),* 97**,** 77-86.

Busch W., Duis K., Fenske M., Maack G., Legler J., Padilla S., Strähle U., Witters H. & Scholz S. 2011. The zebrafish embryo model in toxicology and teratology, September 2–3, 2010, Karlsruhe, Germany. *Reproductive Toxicology,* 31**,** 585-588.

Buske C. & Gerlai R. 2011. Shoaling develops with age in zebrafish (*Danio rerio*). *Progress in Neuro-Psychopharmacology and Biological Psychiatry,* 35**,** 1409-1415.

Cachat J., Stewart A., Grossman L., Gaikwad S., Kadri F., Chung K.M., Wu N., Wong K., Roy S., Suciu C., Goodspeed J., Elegante M., Bartels B., Elkhayat S., Tien D., Tan J., Denmark A., Gilder T., Kyzar E., Dileo J., Frank K., Chang K., Utterback E., Hart P. & Kalueff A.V. 2010. Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. *Nature Protocols,* 5**,** 1786-99.

Cachat J., Stewart A., Utterback E., Hart P., Gaikwad S., Wong K., Kyzar E., Wu N. & Kalueff A.V. 2011. Three-dimensional neurophenotyping of adult zebrafish behavior. *PLoS One,* 6**,** e17597.

Cachat J.M. 2013. *Developing zebrafish models of complex phenotypes relevant to human brain disorders.* PhD dissertation, Tulane University.

Cheng R., Jia Y., Dai L., Liu C., Wang J., Li G. & Yu L. 2017. Tris(1,3-dichloro-2-propyl) phosphate disrupts axonal growth, cholinergic system and motor behavior in early life zebrafish. *Aquatic Toxicology,* 192**,** 7-15.

Christensen J., Grønborg T.K., Sørensen M.J., Schendel D., Parner E.T., Pedersen L.H. & Vestergaard M. 2013. Prenatal valproate exposure and risk of autism spectrum disorders and childhood autism. *JAMA: Journal of the American Medical Association,* 309**,** 1696-1703.

Cianca V., Bartolini T., Porfiri M. & Macri S. 2013. A robotics-based behavioral paradigm to measure anxiety-related responses in zebrafish. *PLoS One,* 8**,** e69661.

Claudio L., Kwa W.C., Russell A.L. & Wallinga D. 2000. Testing methods for developmental neurotoxicity of environmental chemicals. *Toxicology and Applied Pharmacology,* 164**,** 1-14.

Colwill R.M. & Creton R. 2011. Imaging escape and avoidance behavior in zebrafish larvae. *Reviews of the Neurosciences,* 22**,** 63-73.

Dasgupta S., Cheng V., Vliet S.M., Mitchell C.A. & Volz D.C. 2018. Tris (1, 3-dichloro-2-propyl) phosphate exposure during the early-blastula stage alters the normal trajectory of zebrafish embryogenesis. *Environmental Science & Technology,* 52**,** 10820-10828.

De Esch C., Slieker R., Wolterbeek A., Woutersen R. & De Groot D. 2012. Zebrafish as potential model for developmental neurotoxicity testing: A mini review. *Neurotoxicology and Teratology,* 34**,** 545-53.

De Schryver E., De Reu L., Belpaire F. & Willems J. 1987. Toxicokinetics of methyl paraoxon in the dog. *Archives of Toxicology,* 59**,** 319-322.

Di Paolo C., Seiler T.B., Keiter S., Hu M., Muz M., Brack W. & Hollert H. 2015. The value of zebrafish as an integrative model in effect-directed analysis – a review. *Environmental Sciences Europe,* 27.

Dieter H.H. 2014. Health related guide values for drinking-water since 1993 as guidance to assess presence of new analytes in drinking-water. *International Journal of Hygiene and Environmental Health,* 217**,** 117-132.

Dishaw L.V., Hunter D.L., Padnos B., Padilla S. & Stapleton H.M. 2014a. Developmental exposure to organophosphate flame retardants elicits overt toxicity and alters behavior in early life stage zebrafish (*Danio rerio*). *Toxicological Sciences,* 142**,** 445-454.

Dishaw L.V., Macaulay L.J., Roberts S.C. & Stapleton H.M. 2014b. Exposures, mechanisms, and impacts of endocrine-active flame retardants. *Current Opinion in Pharmacology,* 19**,** 125-33.

Dzieweczynski T.L. & Hebert O.L. 2012. Fluoxetine alters behavioral consistency of aggression and courtship in male Siamese fighting fish, *Betta splendens*. *Physiology & Behavior,* 107**,** 92-97.

Dzyadevych S.V., Soldatkin A.P. & Chovelon J.-M. 2002. Assessment of the toxicity of methyl parathion and its photodegradation products in water samples using conductometric enzyme biosensors. *Analytica Chimica Acta,* 459**,** 33-41.

Eckhardt A., Grummt T., Kramer M., Braunbeck T. & Hollert H. 2017. ToxBox – a new approach for evaluating anthropogenic trace substances in drinking water. *Naunyn-Schmiedebergs Archives of Pharmacology,* 390**,** 1-101.

Eddins D., Cerutti D., Williams P., Linney E. & Levin E.D. 2010. Zebrafish provide a sensitive model of persisting neurobehavioral effects of developmental chlorpyrifos exposure: Comparison with nicotine and pilocarpine effects and relationship to dopamine deficits. *Neurotoxicology and Teratology,* 32**,** 99-108.

Egan R.J., Bergner C.L., Hart P.C., Cachat J.M., Canavello P.R., Elegante M.F., Elkhayat S.I., Bartels B.K., Tien A.K., Tien D.H., Mohnot S., Beeson E., Glasgow E., Amri H., Zukowska Z. & Kalueff A.V. 2009. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioural Brain Research,* 205**,** 38-44.

Embry M.R., Belanger S.E., Braunbeck T., Galay-Burgos M., Halder M., Hinton D.E., Léonard M.A., Lillicrap A., Norberg-King T. & Whale G. 2010. The fish embryo toxicity test as an animal alternative method in hazard and risk assessment and scientific research. *Aquatic Toxicology,* 97**,** 79-87.

Engeszer R.E., Patterson L.B., Rao A.A. & Parichy D.M. 2007. Zebrafish in the wild: A review of natural history and new notes from the field. *Zebrafish,* 4**,** 21-40.

Fernandes Y. & Gerlai R. 2009. Long-term behavioral changes in response to early developmental exposure to ethanol in zebrafish. *Alcoholism: Clinical and Experimental Research,* 33**,** 601-609.

Ferrari B., Paxéus N., Giudice R.L., Pollio A. & Garric J. 2003. Ecotoxicological impact of pharmaceuticals found in treated wastewaters: Study of carbamazepine, clofibric acid, and diclofenac. *Ecotoxicology and Environmental Safety,* 55**,** 359-370.

Franks M.E., Macpherson G.R. & Figg W.D. 2004. Thalidomide. *The Lancet,* 363**,** 1802-1811.

Frese L. & Braunbeck T. 2022. Adapting classic paradigms to analyze alterations of shoal-wide behavior in early-life stages of zebrafish (*Danio rerio*) – a case study with fluoxetine. *Neurotoxicology and Teratology,* 95**,** 107136.

Galus M., Kirischian N., Higgins S., Purdy J., Chow J., Rangaranjan S., Li H., Metcalfe C. & Wilson J.Y. 2013. Chronic, low concentration exposure to pharmaceuticals impacts multiple organ systems in zebrafish. *Aquatic Toxicology,* 132-133**,** 200-11.

Gaworecki K.M. & Klaine S.J. 2008. Behavioral and biochemical responses of hybrid striped bass during and after fluoxetine exposure. *Aquatic Toxicology,* 88**,** 207-213.

Gerlai R., Lahav M., Guo S. & Rosenthal A. 2000. Drinks like a fish: Zebra fish (*Danio rerio*) as a behavior genetic model to study alcohol effects. *Pharmacology Biochemistry and Behavior,* 67**,** 773-782.

Gerlai R., Fernandes Y. & Pereira T. 2009. Zebrafish (*Danio rerio*) responds to the animated image of a predator: Towards the development of an automated aversive task. *Behavioural Brain Research,* 201**,** 318-324.

Gerlai R. 2010. Zebrafish antipredatory responses: A future for translational research? *Behavioural Brain Research,* 207**,** 223-231.

Giacomini A.C.V., De Abreu M.S., Koakoski G., Idalencio R., Kalichak F., Oliveira T.A., Da Rosa J.G.S., Gusso D., Piato A.L. & Barcellos L.J.G. 2015. My stress, our stress: Blunted cortisol response to stress in isolated housed zebrafish. *Physiology & Behavior,* 139**,** 182-187.

Grandjean P. & Landrigan P.J. 2006. Developmental neurotoxicity of industrial chemicals. *The Lancet,* 368**,** 2167-2178.

Grosse S.D., Matte T.D., Schwartz J. & Jackson R.J. 2002. Economic gains resulting from the reduction in children's exposure to lead in the United States. *Environmental Health Perspectives,* 110**,** 563-569.

Grummt T., Kuckelhorn J., Bahlmann A., Baumstark-Khan C., Brack W., Braunbeck T., Feles S., Gartiser S., Glatt H., Heinze R., Hellweg C.E., Hollert H., Junek R., Knauer M., Kneib-Kissinger B., Kramer M., Krauss M., Küster E., Maletz S., Meinl W., Noman A., Prantl E.-M., Rabbow E., Redelstein R., Rettberg P., Schadenboeck W., Schmidt C., Schulze T., Seiler T.-B., Spitta L., Stengel D., Waldmann P. & Eckhardt A. 2013. Tox-Box: Securing drops of life - an enhanced health-related approach for risk assessment of drinking water in Germany. *Environmental Sciences Europe,* 25.

Grummt T., Seiler T.-B., Braunbeck T. & Hollert H. 2018a. Editorial: Special issue "effect-related evaluation of anthropogenic trace substances – concepts for genotoxicity, neurotoxicity and endocrine effects". *Environmental Sciences and Pollution Research,* 25**,** 3945-3950.

Grummt T., Braunbeck T., Hollert H. & Kramer M. 2018b. Leitfaden – Gefährdungsbasiertes Risikomanagement für anthropogene Spurenstoffe zur Sicherung der Trinkwasserversorgung (Tox Box). *Umweltbundesamt*, Bad Elster, Germany.

Grummt T., Braunbeck T., Hollert H. & Kramer M. 2020. RiSKWa guideline: Hazard-based risk management of anthropogenic trace substances in drinking water to secure a long-term drinking water supply. Federal Ministry of Education and Research (BMBF).

Halder M., Léonard M., Iguchi T., Oris J.T., Ryder K., Belanger S.E., Braunbeck T., Embry M.R., Whale G., Norberg-King T. & Lillicrap A. 2010. Regulatory aspects on the use of fish embryos in environmental toxicology. *Integrated Environmental Assessment and Management,* 6**,** 484-491.

He J.-H., Gao J.-M., Huang C.-J. & Li C.-Q. 2014. Zebrafish models for assessing developmental and reproductive toxicity. *Neurotoxicology and Teratology,* 42**,** 35-42.

Hutchinson T.H., Wheeler J.R., Gourmelon A. & Burden N. 2016. Promoting the 3Rs to enhance the OECD fish toxicity testing framework. *Regulatory Toxicology and Pharmacology,* 76**,** 231-233.

Jamal G.A., Hansen S. & Julu P.O.O. 2002. Low level exposures to organophosphorus esters may cause neurotoxity. *Toxicology***,** 23-33.

Johnson R., Wolf J. & Braunbeck T. 2009. OECD guidance document for the diagnosis of endocrine-related histopathology of fish gonads. *Organization for Economic Co-operation and Development,* 96.

Kais B., Stengel D., Batel A. & Braunbeck T. 2015. Acetylcholinesterase in zebrafish embryos as a tool to identify neurotoxic effects in sediments. *Environmental Sciences and Pollution Research,* 22**,** 16329-16339.

Kalueff A.V., Stewart A.M. & Gerlai R. 2014. Zebrafish as an emerging model for studying complex brain disorders. *Trends in Pharmacological Sciences,* 35**,** 63-75.

Kanungo J., Cuevas E., Ali S.F. & Paule M.G. 2014. Zebrafish model in drug safety assessment. *Current Pharmaceutical Design,* 20**,** 5416-5429.

Kokel D., Bryan J., Laggner C., White R., Cheung C.Y.J., Mateus R., Healey D., Kim S., Werdich A.A., Haggarty S.J., Macrae C.A., Shoichet B. & Peterson R.T. 2010. Rapid behavior-based identification of neuroactive small molecules in the zebrafish. *Nature Chemical Biology,* 6**,** 231-237.

Kokel D. & Peterson R.T. 2011. Using the zebrafish photomotor response for psychotropic drug screening. *Methods Cell Biol,* 105**,** 517-24.

Kroes R., Galli C., Munro I., Schilter B., Tran L.-A., Walker R. & Würtzen G. 2000. Threshold of toxicological concern for chemical substances present in the diet: A practical tool for assessing the need for toxicity testing. *Food and Chemical Toxicology,* 38**,** 255-312.

Kuckelkorn J., Frese L., Schuller C., Grundlach M., Haigis A.-C., Ogungbemi A., Waldmann P. & Weiß S. 2020. Projekt NeuroBox – wie beeinflusst Wasser unser Nervensystem? *Vom Wasser,* 118**,** 1-32.

Kulkarni P., Chaudhari G.H., Sripuram V., Banote R.K., Kirla K.T., Sultana R., Rao P., Oruganti S. & Chatti K. 2014. Oral dosing in adult zebrafish: Proof-of-concept using pharmacokinetics and pharmacological evaluation of carbamazepine. *Pharmacological Reports,* 66**,** 179-83.

Küster E. & Altenburger R. 2006. Comparison of cholin- and carboxylesterase enzyme inhibition and visible effects in the zebra fish embryo bioassay under short-term paraoxon-methyl exposure. *Biomarkers,* 11**,** 341-54.

Kwon J.-W. & Armbrust K.L. 2006. Laboratory persistence and fate of fluoxetine in aquatic environments. *Environmental Toxicology and Chemistry,* 25**,** 2561-2568.

Kysil E.V., Meshalkina D.A., Frick E.E., Echevarria D.J., Rosemberg D.B., Maximino C., Lima M.G., Abreu M.S.D., Giacomini A.C.V., Barcellos L.J.G., Song C. & Kalueff A.V. 2017. Comparative analyses of zebrafish anxiety-like behavior using conflict-based novelty tests. *Zebrafish,* 14**,** 197-208.

Ladu F., Bartolini T., Panitz S.G., Chiarotti F., Butail S., Macrì S. & Porfiri M. 2015. Live predators, robots, and computer-animated images elicit differential avoidance responses in zebrafish. *Zebrafish,* 12**,** 205-214.

Lammer E. 2009. *Refinement of the fish embryo toxicity test (FET) with zebrafish (Danio rerio): Is it a real replacement of the acute fish toxicity test?* PhD Dissertation, Heidelberg.

Lammer E., Carr G.J., Wendler K., Rawlings J.M., Belanger S.E. & Braunbeck T. 2009. Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? *Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology,* 149**,** 196-209.

Latendresse J.R., Warbrittion A.R., Jonassen H. & Creasy D.M. 2002. Fixation of testes and eyes using a modified Davidson's fluid: Comparison with Bouin's fluid and conventional Davidson's fluid. *Toxicologic Pathology,* 30**,** 524-33.

Legradi J., Di Paolo C., Kraak M., Van Der Geest H., Schymanski E., Williams A., Dingemans M., Massei R., Brack W. & Cousin X. 2018. An ecotoxicological view on neurotoxicity assessment. *Environmental Sciences Europe,* 30**,** 1-34.

Levin E.D., Bencan Z. & Cerutti D.T. 2007. Anxiolytic effects of nicotine in zebrafish. *Physiology & Behavior,* 90**,** 54-58.

Luca R.M. & Gerlai R. 2012. In search of optimal fear inducing stimuli: Differential behavioral responses to computer animated images in zebrafish. *Behavioural Brain Research,* 226**,** 66-76.

Macdonald R.L. 1989. Antiepileptic drug actions. *Epilepsia,* 30**,** S19-S28.

Martins E.P. & Bhat A. 2014. Population-level personalities in zebrafish: Aggression-boldness across but not within populations. *Behavioral Ecology,* 25**,** 368-373.

Maruya K.A., Dodder N.G., Sengupta A., Smith D.J., Lyons J.M., Heil A.T. & Drewes J.E. 2016. Multimedia screening of contaminants of emerging concern (CECS) in coastal urban watersheds in southern California (USA). *Environmental Toxicology and Chemistry,* 35**,** 1986-1994.

Maximino C., Marques De Brito T., Dias F., Cortes F.V., Taccolini I.B., Pereira P.M., Colmanetti R., Gazolla R.A., Tavares R.I., Rodrigues S.T.K., Valéria S., Pontes A.a.A., Romao C.F., Prado V.M. & Gouveia A.J. 2007. A comparative analysis of the preference for dark environments in five teleosts. *International Journal of Comparative Psychology,* 20**,** 351-367.

Maximino C., Marques De Brito T., Dias C.a.G.D.M., Gouveia A.J. & Morato S. 2010. Scototaxis as anxiety-like behavior in fish. *Nature Protocols,* 5**,** 209-216.

Maximino C., Benzecry R., Matos Oliveira K.R., De Jesus Oliveira Batista E., Herculano A.M., Broock Rosemberg D., De Oliveira D.L. & Blaser R. 2012. A comparison of the light/dark and novel tank tests in zebrafish. *Behaviour,* 149**,** 1099-1123.

Maximino C., Puty B., Benzecry R., Araujo J., Lima M.G., De Jesus Oliveira Batista E., De Matos Oliveira K.R., Crespo-Lopez M.E. & Herculano A.M. 2013a. Role of serotonin in zebrafish (*Danio rerio*) anxiety: Relationship with serotonin levels and effect of buspirone, WAY 100635, SB 224289, fluoxetine and para-chlorophenylalanine (pcpa) in two behavioral models. *Neuropharmacology,* 71**,** 83-97.

Maximino C., Puty B., Matos Oliveira K.R. & Herculano A.M. 2013b. Behavioral and neurochemical changes in the zebrafish leopard strain. *Genes, Brain and Behavior,* 12**,** 576-582.

Mcgee S.P., Cooper E.M., Stapleton H.M. & Volz D.C. 2012. Early zebrafish embryogenesis is susceptible to developmental TDCPP exposure. *Environmental Health Perspectives,* 120**,** 1585-1591.

Mckim J.M. 1977. Evaluation of tests with early life stages of fish for predicting long-term toxicity. *Journal of the Fisheries Research Board of Canada,* 34**,** 1148-1154.

Metcalfe C.D., Miao X.-S., Koenig B.G. & Struger J. 2003. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. *Environmental Toxicology and Chemistry,* 22**,** 2881-2889.

Miller N. & Gerlai R. 2007. Quantification of shoaling behaviour in zebrafish (*Danio rerio*). *Behavioural Brain Research,* 184**,** 157-166.

Miller N.Y. & Gerlai R. 2008. Oscillations in shoal cohesion in zebrafish (*Danio rerio*). *Behavioural Brain Research,* 193**,** 148-151.

Mole R.A. & Brooks B.W. 2019. Global scanning of selective serotonin reuptake inhibitors: Occurrence, wastewater treatment and hazards in aquatic systems. *Environmental Pollution,* 250**,** 1019-1031.

Mora-Zamorano F.X., Svoboda K.R. & Carvan M.J., 3rd 2016. The nicotine-evoked locomotor response: A behavioral paradigm for toxicity screening in zebrafish (*Danio rerio*) embryos and eleutheroembryos exposed to methylmercury. *PLoS One,* 11**,** e0154570.

Nair A., Nguyen C. & Mchenry M.J. 2017. A faster escape does not enhance survival in zebrafish larvae. *Proceedings of the Royal Society B: Biological Sciences,* 284.

OECD 2012. Fish short term reproduction assay. *OECD*: TG 229.

OECD 2013a. Fish, early-life stage toxicity test. *OECD*: TG 210.

OECD 2013b. Fish embryo acute toxicity (FET) test. *OECD*: TG 236.

Ogungbemi A.O., Teixido E., Massei R., Scholz S. & Küster E. 2020. Optimization of the spontaneous tail coiling test for fast assessment of neurotoxic effects in the zebrafish embryo using an automated workflow in KNIME. *Neurotoxicology and Teratology,* 81**,** 106918.

Oliveri A.N., Bailey J.M. & Levin E.D. 2015. Developmental exposure to organophosphate flame retardants causes behavioral effects in larval and adult zebrafish. *Neurotoxicology and Teratology,* 52**,** 220-7.

Oris J.T., Belanger S.E. & Bailer A.J. 2012. Baseline characteristics and statistical implications for the OECD 210 fish early-life stage chronic toxicity test. *Environmental Toxicology and Chemistry,* 31**,** 370-376.

Orozco-Hernandez J.M., Gomez-Olivan L.M., Elizalde-Velazquez G.A., Rosales-Perez K.E., Cardoso-Vera J.D., Heredia-Garcia G., Islas-Flores H., Garcia-Medina S. & Galar-Martinez M. 2022. Fluoxetine-induced neurotoxicity at environmentally relevant concentrations in adult zebrafish *Danio rerio*. *Neurotoxicology,* 90**,** 121-129.

Oswald M. & Robison B.D. 2008. Strain-specific alteration of zebrafish feeding behavior in response to aversive stimuli. *Canadian Journal of Zoology,* 86**,** 1085-1094.

Padilla S., Hunter D.L., Padnos B., Frady S. & Macphail R.C. 2011. Assessing locomotor activity in larval zebrafish: Influence of extrinsic and intrinsic variables. *Neurotoxicology and Teratology,* 33**,** 624-30.

Pagnussat N., Piato A.L., Schaefer I.C., Blank M., Tamborski A.R., Guerim L.D., Bonan C.D., Vianna M.R.M. & Lara D.R. 2013. One for all and all for one: The importance of shoaling on behavioral and stress responses in zebrafish. *Zebrafish,* 10**,** 338-342.

Paine M.F. 2017. Therapeutic disasters that hastened safety testing of new drugs. *Clinical Pharmacology & Therapeutics,* 101**,** 430-434.

Pandina M.T. 2002. Ghost-It! 1.04 ed. MajorGeeks.com.

Papadopoulou-Mourkidou E., Karpouzas D.G., Patsias J., Kotopoulou A., Milothridou A., Kintzikoglou K. & Vlachou P. 2004. The potential of pesticides to contaminate the groundwater resources of the axios river basin. Part II. Monitoring study in the south part of the basin. *Science of the Total Environment,* 321**,** 147-164.

Parichy D.M. 2015. Advancing biology through a deeper understanding of zebrafish ecology and evolution. *eLIFE,* 4.

Parker M.O., Millington M.E., Combe F.J. & Brennan C.H. 2012. Housing conditions differentially affect physiological and behavioural stress responses of zebrafish, as well as the response to anxiolytics. *PLoS One,* 7**,** e34992.

Parng C., Seng W.L., Semino C. & Mcgrath P. 2002. Zebrafish: A preclinical model for drug screening. *Assay and Drug Development Technologies,* 1**,** 41-48.

Parolini M., Ghilardi A., De Felice B. & Del Giacco L. 2019. Environmental concentration of fluoxetine disturbs larvae behavior and increases the defense response at molecular level in zebrafish (*Danio rerio*). *Environmental Sciences and Pollution Research,* 26**,** 34943-34952.

Pelli M. & Connaughton V.P. 2015. Chronic exposure to environmentally-relevant concentrations of fluoxetine (Prozac) decreases survival, increases abnormal behaviors, and delays predator escape responses in guppies. *Chemosphere,* 139**,** 202-209.

Pfluger P. & Dietrich D.R. 2001. Effects on pharmaceuticals in the environment – an overview and principle considerations. *Pharmaceuticals in the Environment***,** 11-17.

Qiang L., Cheng J., Yi J., Rotchell J.M., Zhu X. & Zhou J. 2016. Environmental concentration of carbamazepine accelerates fish embryonic development and disturbs larvae behavior. *Ecotoxicology,* 25**,** 1426-1437.

Rao J.S., Lee H.J., Rapoport S.I. & Bazinet R.P. 2008. Mode of action of mood stabilizers: Is the arachidonic acid cascade a common target? *Molecular Psychiatry,* 13**,** 585-596.

Rawlings J.M., Belanger S.E., Connors K.A. & Carr G.J. 2019. Fish embryo tests and acute fish toxicity tests are interchangeable in the application of the threshold approach. *Environmental Toxicology and Chemistry,* 38**,** 671-681.

Richards F.M., Alderton W.K., Kimber G.M., Liu Z., Strang I., Redfern W.S., Valentin J.P., Winter M.J. & Hutchinson T.H. 2008. Validation of the use of zebrafish larvae in visual safety assessment. *Journal of Pharmacological and Toxicological Methods,* 58**,** 50-8.

Richendrfer H., Creton R. & Colwill R.M. 2014. The embryonic zebrafish as a model system to study the effects of environmental toxicants on behavior. *In:* Lessmann C.A. & Carver E.A. (eds.) *Zebrafish.* N.Y.: Nova Science Publ.

Rosemberg D.B., Rico E.P., Mussulini B.H.M., Piato A.L., Calcagnotto M.E., Bonan C.D., Dias R.D., Blaser R.E., Souza D.O. & De Oliveira D.L. 2011. Differences in spatio-temporal behavior of zebrafish in the open tank paradigm after a short-period confinement into dark and bright environments. *PLoS One,* 6**,** e19397.

Rufli H. & Springer T.A. 2011. Can we reduce the number of fish in the OECD acute toxicity test? *Environmental Toxicology and Chemistry,* 30**,** 1006-1011.

Russell W.M.S. & Burch R.L. 1959. *The principles of humane experimental technique*, Methuen.

Sackerman J., Donegan J.J., Cunningham C.S., Nguyen N.N., Lawless K., Long A., Benno R.H. & Gould G.G. 2010. Zebrafish behavior in novel environments: Effects of acute exposure to anxiolytic compounds and choice of *Danio rerio* line. *International Journal of Comparative Psychology,* 23**,** 43-61.

Sánchez-Santed F., Cañadas F., Flores P., López-Grancha M. & Cardona D. 2004. Long-term functional neurotoxicity of paraoxon and chlorpyrifos: Behavioural and pharmacological evidence. *Neurotoxicology and Teratology,* 26**,** 305-317.

Santos L.H.M.L.M., Araujo A.N., Fachini A., Pena A., Delerue-Matos C. & Montenegro M.C.B.S.M. 2010. Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. *Journal of Hazardous Materials,* 175**,** 45-95.

Schindelin J., Arganda-Carreras I., Frise E., Kaynig V., Longair M., Pietzsch T., Preibisch S., Rueden C., Saalfeld S., Schmid B., Tinevez J.-Y., White D.J., Hartenstein V., Eliceiri K., Tomancak P. & Cardona A. 2012. Fiji: An open-source platform for biological-image analysis. *Nature Methods,* 9**,** 676-682.

Scholz S., Fischer S., Gundel U., Kuster E., Luckenbach T. & Voelker D. 2008. The zebrafish embryo model in environmental risk assessment – applications beyond acute toxicity testing. *Environmental Sciences and Pollution Research,* 15**,** 394-404.

Selderslaghs I.W.T., Hooyberghs J., De Coen W. & Witters H.E. 2010. Locomotor activity in zebrafish embryos: A new method to assess developmental neurotoxicity. *Neurotoxicology and Teratology,* 32**,** 460-71.

Sharif M.H., Talebnejad M.R., Rastegar K., Khalili M.R. & Nowroozzadeh M.H. 2019. Oral fluoxetine in the management of amblyopic patients aged between 10 and 40 years old: A randomized clinical trial. *Eye,* 33**,** 1060-1067.

Sharma S.K. & Sanghi R. 2012. *Advances in water treatment and pollution prevention*, Springer Netherlands.

Shelton D.S., Shelton S.G., Daniel D.K., Raja M., Bhat A., Tanguay R.L., Higgs D.M. & Martins E.P. 2020. Collective behavior in wild zebrafish. *Zebrafish,* 17**,** 243-252.

Simeon S., Beaudouin R., Brotzmann K., Braunbeck T. & Bois F.Y. 2021. Multistate models of developmental toxicity: Application to valproic acid-induced malformations in the zebrafish embryo. *Toxicology and Applied Pharmacology,* 414**,** 115424.

Sledge D., Yen J., Morton T., Dishaw L., Petro A., Donerly S., Linney E. & Levin E.D. 2011. Critical duration of exposure for developmental chlorpyrifos-induced neurobehavioral toxicity. *Neurotoxicology and Teratology,* 33**,** 742-51.

Spitsbergen J.M. & Kent M.L. 2003. The state of the art of the zebrafish model for toxicology and toxicologic pathology research – advantages and current limitations. *Toxicologic Pathology,* 31**,** 62-87.

Stegeman J., Goldstone J.V. & Hahn M.E. 2010. Perspectives on zebrafish as a model in environmental toxicology. *Fish Physiology: Zebrafish.* Academic Press.

Stein J., Schettler T., Wallinga D. & Valenti M. 2002. In harm's way: Toxic threats to child development. *Developmental and Behavioral Pediatrics,* 23**,** S13-S22.

Stengel D., Zindler F. & Braunbeck T. 2017a. An optimized method to assess ototoxic effects in the lateral line of zebrafish (*Danio rerio*) embryos. *Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology,* 193**,** 18-29.

Stengel D., Wahby S. & Braunbeck T. 2017b. In search of a comprehensible set of endpoints for the routine monitoring of neurotoxicity in vertebrates: Sensory perception and nerve transmission in zebrafish (*Danio rerio*) embryos. *Environmental Sciences and Pollution Research*.

Stewart A., Gaikwad S., Kyzar E., Green J., Roth A. & Kalueff A.V. 2012. Modeling anxiety using adult zebrafish: A conceptual review. *Neuropharmacology,* 62**,** 135-143.

Stewart A.M., Cachat J., Gaikwad S., Robinson K.S.L., Gebhardt M. & Kalueff A.V. 2013. Perspectives on experimental models of serotonin syndrome in zebrafish. *Neurochemistry International,* 62**,** 893-902.

Stewart A.M., Braubach O., Spitsbergen J., Gerlai R. & Kalueff A.V. 2014a. Zebrafish models for translational neuroscience research: From tank to bedside. *Trends in Neurosciences,* 37**,** 264-78.

Stewart A.M., Grossman L., Nguyen M., Maximino C., Rosemberg D.B., Echevarria D.J. & Kalueff A.V. 2014b. Aquatic toxicology of fluoxetine: Understanding the knowns and the unknowns. *Aquatic Toxicology,* 156**,** 269-273.

Strähle U., Scholz S., Geisler R., Greiner P., Hollert H., Rastegar S., Schumacher A., Selderslaghs I., Weiss C., Witters H. & Braunbeck T. 2012. Zebrafish embryos as an alternative to animal experiments--a commentary on the definition of the onset of protected life stages in animal welfare regulations. *Reproductive Toxicology,* 33**,** 128-132.

Sumpter J.P., Donnachie R.L. & Johnson A.C. 2014. The apparently very variable potency of the anti-depressant fluoxetine. *Aquatic Toxicology,* 151**,** 57-60.

Suriyampola P.S., Shelton D.S., Shukla R., Roy T., Bhat A. & Martins E.P. 2016. Zebrafish social behavior in the wild. *Zebrafish,* 13.

Teixido E., Pique E., Gomez-Catalan J. & Llobet J.M. 2013. Assessment of developmental delay in the zebrafish embryo teratogenicity assay. *Toxicology In Vitro,* 27**,** 469-478.

Ternes T. 1998. Occurrence of drugs in German sewage treatment plants and rivers. *Water Research.*

Tilton F.A., Bammler T.K. & Gallagher E.P. 2011. Swimming impairment and acetylcholinesterase inhibition in zebrafish exposed to copper or chlorpyrifos separately, or as mixtures. *Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology,* 153**,** 9-16.

Tisler S., Zindler F., Freeling F., Nödler K., Toelgyesi L., Braunbeck T. & Zwiener C. 2019. Transformation products of fluoxetine formed by photodegradation in water and biodegradation in zebrafish embryos (*Danio rerio*). *Environmental Science & Technology,* 53**,** 7400-7409.

Ton C., Lin Y. & Willett C. 2006. Zebrafish as a model for developmental neurotoxicity testing. *Birth Defects Research Part A: Clinical and Molecular Teratology,* 76**,** 553-567.

Tropepe V. & Sive H.L. 2003. Can zebrafish be used as a model to study the neurodevelopmental causes of autism? *Genes, Brain and Behavior,* 2**,** 268-281.

Truong L., Saili K.S., Miller J.M., Hutchison J.E. & Tanguay R.L. 2012. Persistent adult zebrafish behavioral deficits results from acute embryonic exposure to gold nanoparticles. *Computational Biochemistry and Physiology, Part C: Toxicology and Pharmacology,* 155**,** 269-274.

UBA 2003. Bewertung der Anwesenheit teil- oder nicht bewertbarer Stoffe im Trinkwasser aus gesundheitlicher Sicht: Empfehlung des Umweltbundesamtes nach Anhörung der Trinkwasserkommission beim Umweltbundesamt. *Bundesgesundheitsblatt – Gesundheitsforschung – Gesundheitsschutz,* 46**,** 249-251.

Van Der Veen I. & De Boer J. 2012. Phosphorus flame retardants: Properties, production, environmental occurrence, toxicity and analysis. *Chemosphere,* 88**,** 1119-1153.

Vargesson N. 2015. Thalidomide-induced teratogenesis: History and mechanisms. *Birth Defects Research Part C: Embryo Today: Reviews,* 105**,** 140-156.

Vargesson N. 2019. The teratogenic effects of thalidomide on limbs. *Journal of Hand Surgery (European Volume),* 44**,** 88-95.

Vaughan M. & Van Egmond R. 2010. The use of the zebrafish (*Danio rerio*) embryo for the acute toxicity testing of surfactants, as a possible alternative to the acute fish test. *Alternatives To Laboratory Animals,* 38**,** 231-238.

Von Hellfeld R., Ovcharova V., Bevan S., Lazaridi M.-A., Bauch C., Walker P., Hougaard Bennekou S., Forsby A. & Braunbeck T. 2022. Zebrafish embryo neonicotinoid developmental neurotoxicity in the FET test and behavioral assays. *ALTEX*.

Walker C.H. 2003. Neurotoxic pesticides and behavioural effects upon birds. *Ecotoxicology,* 12**,** 307-316.

Wang C., Chen H., Li H., Yu J., Wang X. & Liu Y. 2020. Review of emerging contaminant tris(1,3-dichloro-2-propyl)phosphate: Environmental occurrence, exposure, and risks to organisms and human health. *Environment International,* 143**,** 105946.

Wang Q., Lam J.C.W., Han J., Wang X., Guo Y., Lam P.K.S. & Zhou B. 2015. Developmental exposure to the organophosphorus flame retardant tris(1,3-dichloro-2-propyl) phosphate: Estrogenic activity, endocrine disruption and reproductive effects on zebrafish. *Aquatic Toxicology,* 160**,** 163-171.

Weigt S., Huebler N., Strecker R., Braunbeck T. & Broschard T.H. 2011. Zebrafish (*Danio rerio*) embryos as a model for testing proteratogens. *Toxicology,* 281**,** 25-36.

Williams E.S., Panko J. & Paustenbach D.J. 2009. The European union's REACH regulation: A review of its history and requirements. *Critical Reviews in Toxicology,* 39**,** 553-175.

Winter M.J., Redfern W.S., Hayfield A.J., Owen S.F., Valentin J.-P. & Hutchinson T.H. 2008. Validation of a larval zebrafish locomotor assay for assessing the seizure liability of early-stage development drugs. *Journal of Pharmacological and Toxicological Methods,* 57**,** 176-187.

Wlodkowic D., Bownik A., Leitner C., Stengel D. & Braunbeck T. 2022. Beyond the behavioural phenotype: Uncovering mechanistic foundations in aquatic eco-neurotoxicology. *Science of the Total Environment,* 829**,** 154584.

Wong D.T., Horng J.S., Bymaster F.P., Hauser K.L. & Molloy B.B. 1974. A selective inhibitor of serotonin uptake: Lilly 110140, 3-(p-trifluoromethylphenoxy)-n-methyl-3-phenylpropylamine. *Life Sciences,* 15**,** 471-479.

Wong K., Elegante M., Bartels B., Elkhayat S., Tien D., Roy S., Goodspeed J., Suciu C., Tan J., Grimes C., Chung A., Rosenberg M., Gaikwad S., Denmark A., Jackson A., Kadri F., Chung K.M., Stewart A., Gilder T., Beeson E., Zapolsky I., Wu N., Cachat J. & Kalueff A.V. 2010. Analyzing habituation responses to novelty in zebrafish (*Danio rerio*). *Behavioural Brain Research,* 208**,** 450-457.

World health organization 2011. Guidelines for drinking-water quality (fourth edition). *World health organization,* Geneva.

Zhang Y., Geißen S.-U. & Gal C. 2008. Carbamazepine and diclofenac: Removal in wastewater treatment plants and occurrence in water bodies. *Chemosphere,* 73**,** 1151-1161.

Zindler F., Beedgen F. & Braunbeck T. 2019a. Time-course of coiling activity in zebrafish (*Danio rerio*) embryos exposed to ethanol as an endpoint for developmental neurotoxicity (DNT) – hidden potential and underestimated challenges. *Chemosphere,* 235**,** 12-20.

Zindler F., Beedgen F., Brandt D., Steiner M., Stengel D., Baumann L. & Braunbeck T. 2019b. Analysis of tail coiling activity of zebrafish (*Danio rerio*) embryos allows for the differentiation of neurotoxicants with different modes of action. *Ecotoxicology and Environmental Safety,* 186**,** 109754.

Zindler F., Tisler S., Loerracher A.-K., Zwiener C. & Braunbeck T. 2020a. Norfluoxetine is the only metabolite of fluoxetine in zebrafish (*Danio rerio*) embryos that accumulates at environmentally relevant exposure scenarios. *Environmental Science & Technology,* 54**,** 4200-4209.

Zindler F., Stoll S., Baumann L., Knoll S., Huhn C. & Braunbeck T. 2020b. Do environmentally relevant concentrations of fluoxetine and citalopram impair stress-related behavior in zebrafish (*Danio rerio*) embryos? *Chemosphere,* 261**,** 127753.